On the origin of growth-associated protein-43 (GAP-43) immunoreactive processes present in the rat anterior pituitary

by Jason Michael Kuhl

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Montana State University

Abstract:

Recent studies have demonstrated the presence of an axon plexus immunoreactive (ir) for the neuronal growth-associated phosphoprotein-43 (GAP-43), a membrane-bound phosphoprotein implicated in axonal growth, within the rat anterior pituitary (AP). Though these fibers are implicated in modulating the hypothalamo-pituitary-adrenal (HPA) axis, their source of origin remains unknown. The current studies used anterograde and retrograde neural tracing, surgical ablation, and immunocytochemistry in an effort to elucidate the source(s) of GAP-43-ir processes in the adult male rat AP. In the first experiment the lipophilic tracer dialkylcarbocyanine (DiI) was used to determine if the GAP-43-ir processes were originating from the trigeminal ganglia, and therefore of sensory origin. Due to diffusion of the DiI and very little labeling of structures in the AP this study was determined to be inconclusive: The second experiment looked at possible autonomic innervation, and involved the bilateral removal of the superior cervical ganglia (SCGX). Immunocytochemical staining for GAP-43 after SCGX revealed no decrease in the density of immunoreactive structures, strongly suggesting that the GAP-43-ir structures are not sympathetic in nature. The last two experiments involved retrograde tracing of the AP using either DiI or Fluorogold (FG). DiI tracing, again, appeared to be inconclusive due to dye diffusion. However, retrograde tracing using FG was accomplished, and revealed specific labeling of neuronal cell bodies within the medial habenular nuclei (MHb). This staining does not appear to be artifactual. If existence of this novel central projection is confirmed, it would provide new insights into the mechanisms by which the MHb modulates the stress response.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Recent studies have demonstrated the presence of an axon plexus immunoreactive (ir) for the neuronal growth-associated phosphoprotein-43 (GAP-43), a membrane-bound phosphoprotein implicated in axonal growth, within the rat anterior pituitary (AP). Though these fibers are implicated in modulating the hypothalamo-pituitary-adrenal (HPA) axis, their source of origin remains unknown. The current studies used anterograde and retrograde neural tracing, surgical ablation, and immunocytochemistry in an effort to elucidate the source(s) of GAP-43-ir processes in the adult male rat AP. In the first experiment the lipophilic tracer dialkylcarbocyanine (DiI) was used to determine if the GAP-43-ir processes were originating from the trigeminal ganglia, and therefore of sensory origin. Due to diffusion of the DiI and very little labeling of structures in the AP this study was determined to be inconclusive. The second experiment looked at possible autonomic innervation, and involved the bilateral removal of the superior cervical ganglia (SCGX). Immunocytochemical staining for GAP-43 after SCGX revealed no decrease in the density of immunoreactive structures, strongly suggesting that the GAP-43-ir structures are not sympathetic in nature. The last two experiments involved retrograde tracing of the AP using either DiI or Fluorogold (FG). DiI tracing, again, appeared to be inconclusive due to dye diffusion. However, retrograde tracing using FG was accomplished, and revealed specific labeling of neuronal cell bodies within the medial habenular nuclei (MHb). This staining does not appear to be artifactual. If existence of this novel central projection is confirmed, it would provide new insights into the mechanisms by which the MHb modulates the stress response.

INTRODUCTION

Previous studies done by Paden et al (1994) have described the presence of an extensive plexiform network of fine beaded nerve fibers within the parenchyma of the anterior lobe (AP) of the rat pituitary. These axons were visualized through peroxidase immunocytochemical localization of the neural-specific growth-associated protein-43 (GAP-43, also known as B-50, F1, pp46, p57, and neuromodulin), a membrane-bound phosphoprotein found in axonal growth cones, and implicated in axonogenesis and synaptic remodeling (Benowitz and Perrone-Bizzozero, 1991; Gispen et al., 1991; Meiri et al., 1986; Skene, 1989). The presence of GAP-43 suggested that the axon terminals in the AP are capable of morphological plasticity. Supporting this hypothesis, Lu et al (1995) reported that the density of GAP-43 immunoreactive (GAP-43-ir) axons in the rat adenohypophysis was significantly increased 4 days after bilateral removal of the adrenal glands (ADX). Subsequently, this laboratory demonstrated that the increase in GAP-43-ir was indicative of axonal collateral sprouting as fibers grew to specifically contact over 90% of corticotrophs two weeks after ADX, when these cells are known to be mitotically active and hypertrophic (Paden et al., 1998). The extent, celerity, and selectivity of axonal sprouting in response to ADX strongly suggests that GAP-43-ir axons are of functional significance in the AP, and likely involved in the regulation of the stress response produced by the activation of the hypothalamo-pituitary-adrenal (HPA) axis. However, because GAP-43 is found in a wide variety of nerve fibers in both the central

nervous system (CNS) and peripheral nervous system (PNS) (Bendotti et al., 1991; Benowitz et al., 1988; Del Fiacco et al., 1994; Kruger et al., 1993; Sharkey et al., 1990; Stewart et al., 1992; Yao et al., 1993), its presence within axons innervating the AP reveals little about their possible origin.

The specific goal of this project was to determine the origin of GAP-43-ir axons found in the parenchyma of the rat AP through the use of four different experimental designs. The first study involved post-mortem anterograde tract tracing of the trigeminal nerve (CN V) using the fluorescent dialkylcarbocyanine lipophilic tracer DiI. This experiment was undertaken to test the hypothesis that the GAP-43-ir fibers were sensory in origin. The second experiment involved the bilateral surgical removal of the superior cervical ganglia (SCGX) in an effort to determine if the GAP-43-ir fibers were of sympathetic origin. The third experimental design utilized DiI for post-mortem retrograde tracing of the axons terminating in the rat AP to determine if the GAP-43-ir fibers originated in the brain. The fourth experiment involved retrograde axonal tracing after iontophoresis of Fluorogold (FG) into the ventral AP of live rats, in order to investigate all three previously described potential sources of fibers.

Upon completion of the fourth experiment FG was found to be bilaterally localized within the medial habenular nuclei (MHb). This labeling does not appear to be artifactual, with FG specifically localized within neuronal cell bodies. Furthermore, new evidence suggesting that the MHb is involved in the stress response (Andres et al., 1999; Scheibel, 1997), suggests a possible link with previous studies from this laboratory showing that increased density of GAP-43-ir in the AP is associated with activation of the HPA axis and increased corticotropic activity (Paden et al., 1998).

LITERATURE REVIEW

Development of the Pituitary

The hypophysis (pituitary gland) forms during development from two separate primordial ectodermal outgrowths just beneath the brain at the level of the mesodiencephalic junction. Rathke's pocket is one of these outgrowths, arising from the stomodeal ectoderm of the buccal cavity. The other primordial outgrowth is the infundibular process, arising from the neural ectoderm in the floor of the diencephalon. Rathke's pocket grows along the midline toward the diencephalic floor to secondarily unite with the infundibular process at the level of the diencephalon.

In time the infundibular process develops into the constitutive pars neuralis (neurohypophysial subunit, or neural lobe) of the adult pituitary. Concomitantly, Rathke's pocket grows into a double-layered cup, partially encapsulating the infundibular process of the future pars neuralis. Later in development, the anterior portion of this cup thickens to form the sinusoidal tissue and vasculature known collectively as the pars distalis (adenohypophysial subunit, or anterior lobe) giving rise to the glandular-like appearance of the pituitary. The thin layer of Rathke's pocket adjacent to the pars neuralis ends up forming the last of the three lobes present in the adult organism and is respectively named the pars intermedia (intermediate lobe).

The neural stalk that joins the pituitary gland to the brain is formed mainly by the axons of the vasopressin (AVP) and oxytocin (OX) magnocellular secretory neurons originating in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. The vast majority of AVP and OX axons come to terminate in the neural lobe. In fact, this innervation is so prevalent that the morphology of the developing neural lobe is almost completely characterized by the arborization of these magnocellular axons, along with a relatively small number of glia (pituicytes and microglia) and a rich bed of fenestrated capillaries. In contrast, the histology of the developing anterior lobe consists of six secretory cell types that are capable of producing at least eight separate hormones, supporting glial-like folliculostellate cells, and a dense capillary plexus.

The Functional Pituitary and the Hypothalamic-Pituitary-Adrenal Axis

The neural lobe, intermediate lobe, and anterior lobe have unique and very distinct functions in both the developing and adult mammalian organism. The neural lobe forms a neurosecretory tissue releasing the polypeptides AVP (also known as antidiuretic hormone) and OX. The intermediate lobe secretes melanocyte-stimulating hormone and beta-endorphin. The anterior lobe is composed of a variety of secretory cell types which include: somatotrophs (growth hormone), lactotrophs (prolactin), corticotrophs (adrenocorticotropic hormone), gonadotrophs (lutenizing hormone and follicle stimulating hormone), and thyrotrophs (thyroid stimulating hormone) (Bennett and Whitehead 1983; Nakane, 1970). All eight of the hormones produced by the secretory cells found in the anterior lobe act as tropic effectors for other endocrine

glands, and are involved in the positive and negative feedback loops that assist in the modulation and/or maintenance of an organism's internal environment.

Of particular relevance to this study are the negative feedback loops involved in regulation of the HPA axis (Figure 1). This feedback system regulates the levels of glucocorticoids (corticosterone in rodents, cortisol in most other mammals) found in mammals. Increased activity of the HPA axis is initiated by the hypothalamic release of corticotropin-releasing factor (CRF) in response to physiological and/or environmental stress. The secreted CRF enters the AP via the portal vasculature of the pars tuberalis, thereby functioning as an activator for the secretion of adrenocorticotrophin (ACTH) by the AP. The release of ACTH into the circulatory system, in turn, stimulates the production and secretion of gluococorticoids by the adrenal gland. The feedback loop is completed by the increased systemic levels of glucocorticoids stopping the hypothalamic release of CRF, leading to the negative feedback inhibition of ACTH release. Glucocorticoid negative feedback is also exerted directly at the level of the corticotrophs.

The HPA axis and its control over glucocorticoid levels is of notable physiological importance to the mammalian organism. The corticosteriods secreted during stress exert powerful anti-insulin effects on protein and carbohydrate metabolism, induce synthesis of glucose by the liver, activate enzymes in muscle that are involved in the catabolism of proteins, stimulate lipolysis in adipose tissues, and suppress the immune system (Porth, 1990).

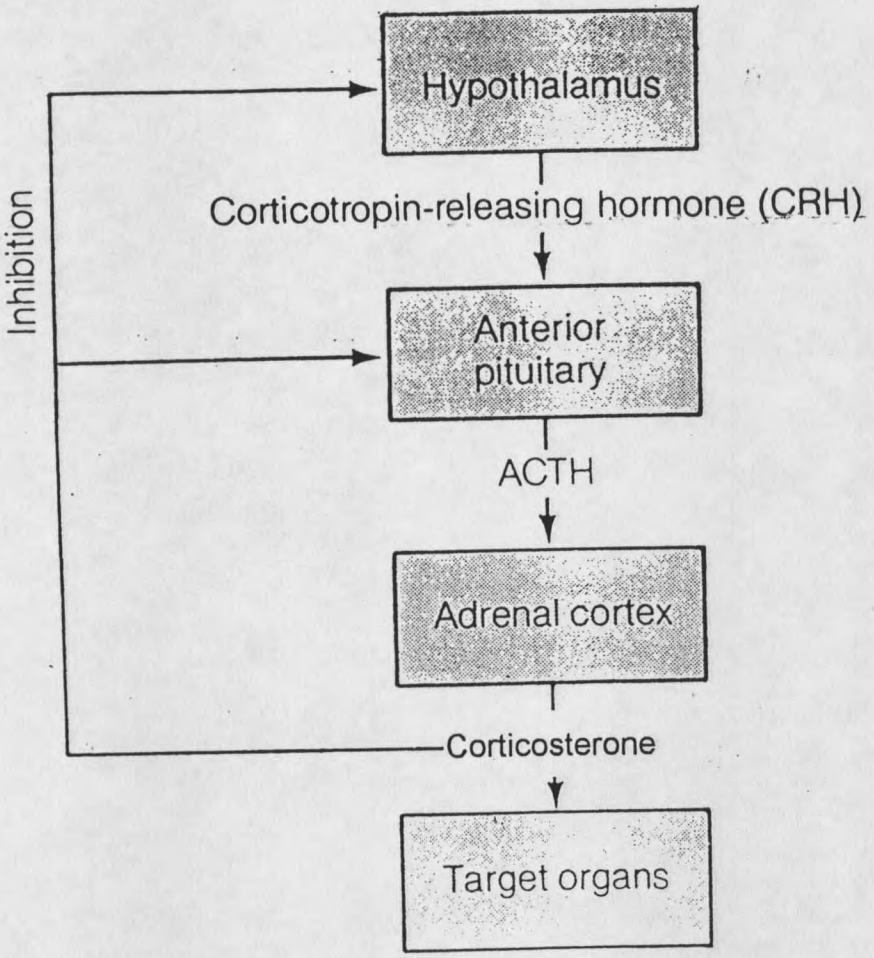

Figure 1. The hypothalamic-pituitary-adrenal (HPA) feedback system regulating circulating glucocorticoid (corticosterone) levels. Increased corticosterone levels incite a negative feedback inhibition of ACTH release by the AP.

During periods of chronic stress the HPA axis of rats is altered, resulting in tonic elevations of plasma corticosterone of varying magnitude (Dallman 1993). Chronic stress is also accompanied by a slightly elevated level of plasma ACTH, increased hypothalamic CRF, and decreased pituitary CRH receptors (Tizabi and Aguilera 1992). Interestingly, the increased level of plasma corticosterone does not inhibit the HPA axis, which is at least fully responsive, if not hypersensitive, to acute or novel stressors (Dallman 1993; Tizabi and Aguilera 1992). The maintenance of HPA axis sensitivity during sustained chronic stress is currently believed to be due to stress induced changes in the input to CRH neurons and in the neurons themselves.

Innervation of the Anterior Pituitary

Throughout most of the 20th century it had been thought that the anterior pituitary was only sparsely innervated by autonomic nerve fibers, and by a few additional fibers of unknown origin (Aleshin, 1964; Dandy, 1913; Friedgood, 1970; Green, 1966). The sparse innervation of the adenohypophysis by the autonomic nervous system is believed to consist mainly of postganglionic ascending fibers from the superior cervical ganglia (SCG). These fibers have been reported to directly innervate the vascular and parenchymatous elements of the AP by way of the cavernous plexus (Friedgood, 1970). It has also been shown that in canines and felines, sympathetic nerve fibers extend off the carotid plexus to innervate the adenohypophysial subunit (Dandy, 1913).

More recently, since the advent of immunocytochemistry, the innervation of the mammalian anterior pituitary gland has been reinvestigated. Convergent work from several groups demonstrated a fairly substantial innervation of the AP by peptidergic nerve fibers in several mammalian species. Currently the rat AP is believed to be innervated by somatostatin fibers (Westlund et al., 1983), serotonin fibers (Westlund and Childs, 1982), and pituitary adenylate cyclase activating peptide (PACAP) fibers (Mikkelsen et al., 1995), while fibers immunoreactive for both substance P (SP) and calcitonin gene-related peptide (CGRP) have been found present in the AP of rats, dogs, macaques, and humans (Gon et al., 1990; Ju et al., 1991; Ju et al., 1993; Ju and Liu, 1989a; Ju and Liu, 1990; Ju and Zhang, 1990; Ju and Zhang, 1992; Liu and Ju, 1988; Lu et al., 1995; Mikkelsen et al., 1989; Skofitsch and Jacobowitz, 1985; Tschopp et al., 1985). Another set of processes immunoreactive for GAP-43 have also been localized to the AP of the rat (Lu et al., 1995; Paden et al., 1994; Paden et al., 1998). However, their exact phenotype has yet to be elucidated and doing so is one of the goals of this project. Many of these fibers have been found to be in close contact with a variety of AP gland cells including lactotrophs, somatotrophs, corticotrophs, gonadotrophs, and thyrotrophs (Gon et al., 1990; Ju et al., 1991; Ju and Liu, 1989b; Liu et al., 1996; Mikkelsen et al., 1989; Paden et al., 1994; Paden et al., 1998; Westlund et al., 1983). Furthermore, electron microscopy has been used to show that some of these fibers form synaptic contacts with the AP secretory cells (Ju and Zhang, 1990; Ju and Zhang, 1992; Liu et al., 1996).

GAP-43 as a Marker of Innervation and Collateral Axonal Sprouting

GAP-43 (also known as B-50, F1, pp46, p57, and neuromodulin) is a 24 kD membrane-associated phosphoprotein present in the axonal growth cones of a wide variety of nerve fibers found throughout both the CNS and PNS (Benowitz et al., 1988; Del Fiacco et al., 1994; Sharkey et al., 1990; Stewart et al., 1992). It is expressed at high levels during the development of the nervous system and is increased during periods of axonal regeneration and morphological plasticity in adults (Benowitz and Perrone-Bizzozero, 1991; Gispen et al., 1991; Meiri et al., 1986; Skene, 1989). The temporal correlation existing between axonal outgrowth and increased GAP-43 expression suggests that GAP-43 plays a functional role in axonogenesis, maintenance of the functional growth cone, and synaptic remodeling (Benowitz and Perrone-Bizzozero, 1991; Benowitz et al., 1981; Gispen et al., 1991; Meiri et al., 1986; Skene, 1989; Skene and Willard, 1981a; Skene and Willard, 1981b; Skene and Willard, 1981c).

Special interest in the function of GAP-43, as well as other growth-associated proteins, has arisen in response to the observation that regeneration and/or sprouting is often accompanied by a specific 20- to 100-fold increase in synthesis of these proteins, and their subsequent axoplasmic transport into the growing axon (Kalil and Skene, 1986; Skene and Willard, 1981a). The presence of GAP-43 within certain areas of the damaged CNS is of particular significance because axoplasmic transport is not normally found to be restored in the injured axons--a likely factor in the inability of most CNS neurons to regenerate and/or undergo morphological changes. Therefore, the re-institution of axoplasmic transport of GAP-43, and its presence in the axonal growth cone in response

to nerve insult in both the CNS and PNS makes GAP-43 an ideal marker for studying the sprouting and regenerative capacity of axons in the nervous system.

Little is known about the true functional significance of GAP-43 within the mammalian nervous system above and beyond its localization to the membrane fraction of the growth cone (it was not found to be present in the cytoskeleton) (Meiri and Gordon-Weeks, 1990). GAP-43 has been shown to behave like an integral membrane protein in detergents and salts (Skene and Willard, 1981a; Dosemeci and Rodnight, 1987). However, the primary sequence of GAP-43 contains no membrane-spanning domains (Basi et al., 1987, Karns et al., 1987). It has been observed that GAP-43 is highly phosphorylated within the neuron when the cell is undergoing depolarization and exocytosis (Dekker et al., 1990; Van Hooff et al., 1989). Furthermore, the kinase C phosphorylation of GAP-43 appears spatially restricted to the growth cone after the protein has undergone axoplasmic transport in its dephosphorylated state (Meiri et al., 1991). Thus there is believed to exist a temporal delay between the onset of GAP-43 expression concurrent with initiation of axonal outgrowth, and the subsequent phosphorylation of GAP-43 believed to be involved in stabilizing the growth cone. Currently it is thought that the induction of GAP-43 is a critical step involved in the conversion of a neuron to a competent metabolic state in which it is capable of axonal elongation (Skene and Willard, 1981b; Skene and Willard, 1981a). If this is indeed occurring, failure of neurons to elevate their levels of GAP-43 protein in response to insult or injury would be a limiting factor in the neuron's ability to regenerate and/or sprout an axon.

GAP-43 has been over-expressed in the neurons of adult transgenic mice (Aigner and Caroni, 1995). Axonal sprouting and terminal arborization were seen to be greatly potentiated compared to normal mice in response to paralyzing the neuromuscular junction, crushing the sciatic nerve, or lesioning the dorsal root ganglia (DRG). However, these transgenic mice also exhibited spontaneous sprouting of axon collaterals at the neuromuscular junction and in the terminal field of hippocampal mossy fibers (Aigner and Caroni, 1995). The hyperphysiological presence of neuronal GAP-43 in the transgenic model is believed to assist in stabilizing spontaneous lamellar and filopodial structures at the axonal terminal (Robbins and Polak, 1988), thereby causing the accumulation of new nerve sprouts in an environment normally devoid of such activity. Thus, it could be hypothesized that up-regulation of GAP-43 in response to injury in a normal organism could also act to stabilize spontaneously forming lamellar and filopodial processes in the regenerating axon.

GAP-43 in the Anterior Pituitary

Earlier studies from this laboratory have demonstrated the presence of an axonal plexus immunoreactive for GAP-43 in the rat AP (Paden et al., 1994). At the level of the light microscope the staining appears as large varicose nerve bundles, likely made up of multiple smaller axons, universally scattered throughout the lobe (Figure 2). Numerous fine beaded axon processes can be seen to course individually through the coronally cut Vibratome sections. However, the majority of the GAP-43 immunoreactivity

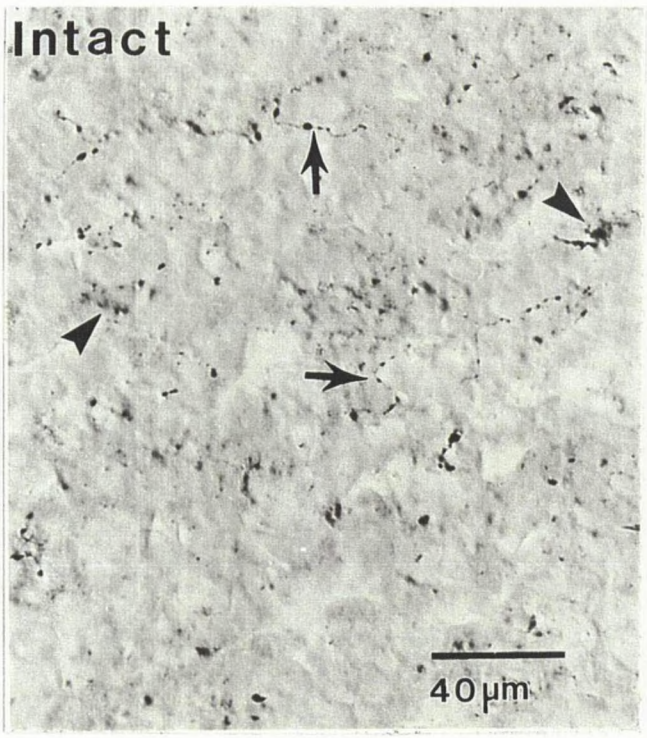

Figure 2. GAP-43 immunoreactivity in a peroxidase stained 40 µm thick section of the intact rat AP. Both fine beaded axons (arrows) and punctate clusters of putative axon terminals (arrowheads) are visible. Magnification is 450X.

(GAP-43-ir) appears as intense punctate staining apparently surrounding a population of secretory cells within the AP. As previously described, the presence of GAP-43 strongly suggests that these apparent axonal terminals are capable of morphological plasticity.

In support of this hypothesis, the density of GAP-43-ir axons in the rat AP is significantly increased 4 days after bilateral adrenalectomy (ADX) (Figure 3 and Lu et al., 1995). Subsequently this laboratory demonstrated that the increase in GAP-43-ir appeared indicative of axonal sprouting, as fibers grew to specifically contact over 90% of corticotrophs two weeks after ADX (Figure 4), a time when these cells are mitotically active and hypertrophic (Paden et al., 1998). The extent and selectivity of the apparent collateral sprouting in contacting corticotrophs strongly suggest that these fibers are of functional significance, giving rise to the hypothesis that the innervation of corticotrophs by GAP-43-ir processes may act as an additional control mechanism that is highly plastic in response to activation of the HPA axis (see below).

Since GAP-43 has previously been shown to facilitate neurotransmitter release by axons (Dekker et al., 1989; Ivins et al., 1993), it could be speculated that these processes are somehow involved in modulating the secretion of ACTH. The fact that plasticity appears as the rule rather than the exception in the HPA axis is of great importance to understanding the physiological mechanisms involved in controlling and modulating stress. The ability of the GAP-43-ir fibers found present in the AP to undergo morphological plasticity would seem to be a requisite for effective innervation of the constantly changing population of corticotrophs.

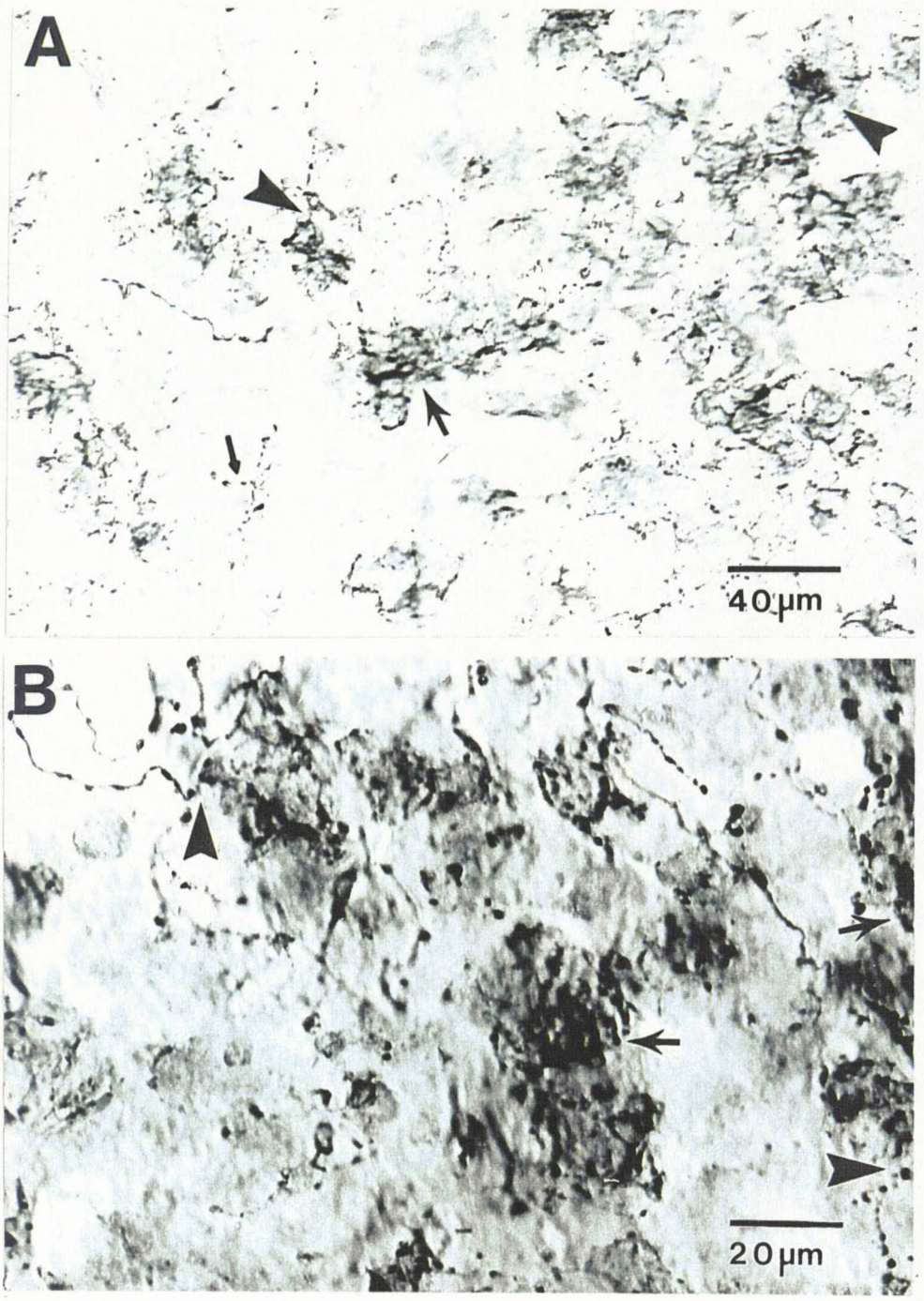

Figure 3. A sustained increase in GAP-43-ir following ADX is apparent in peroxidase stained 40 µm thick sections of the rat AP. **(A)** Post-ADX tissue at 400X magnification; **(B)** post-ADX tissue at 800X magnification. Large groups of targeted cells are frequently seen post-ADX (large arrows). Axons (arrowheads) are often seen entering clusters of terminals surrounding either individual gland cells or groups of cells. Individual axons are also occasionally visible branching from larger axon fascicles (small arrow, **A**).

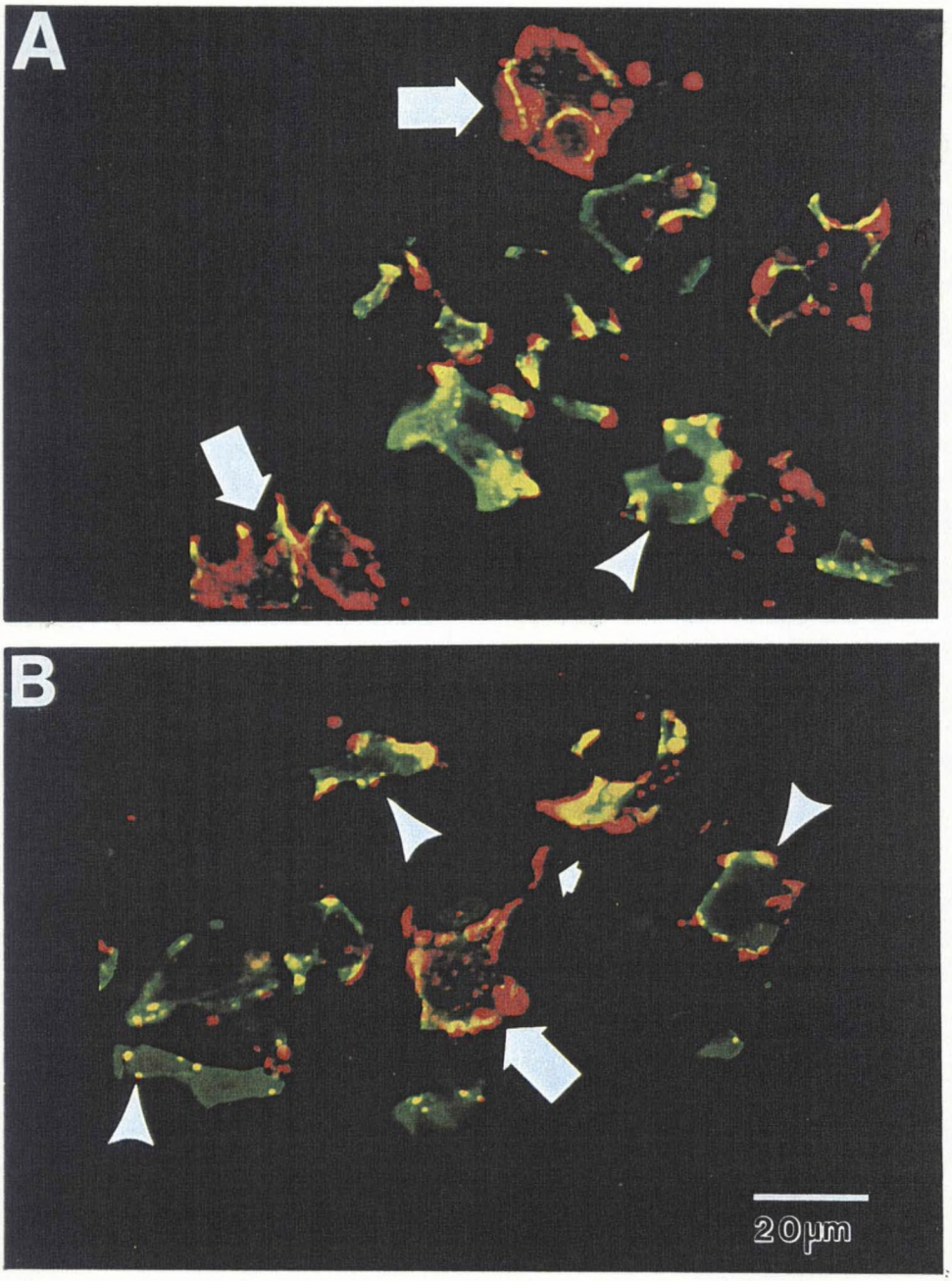

Figure 4. Shown are confocal micrographs of the AP from post-ADX rats. Corticotrophs are identified by green (fluorescein) labeling of ACTH; GAP-43-ir axons by red (Cy3) staining, and areas of overlap are indicated by yellow. Corticotrophs (both individual cells and clusters) are frequently enveloped by GAP-43-ir processes (large arrows); a single process can be seen branching to form a cluster of putative terminals overlying two corticotrophs in **B** (small arrow). GAP-43-ir lying closest to the plane of focus is very punctate and can be seen to be preferentially associated with the cell surface in **A** and **B** (arrowheads). Magnification is 800X.

Potential Sources of the GAP-43-ir AP Innervation

Most of the previously described afferents present in the mammalian AP do not resemble the distribution of GAP-43-ir fibers in the AP. GAP-43 is seen to have an ubiquitous presence throughout the AP, while serotonin and somatostatin fibers are confined primarily to the periphery (Westlund and Childs, 1982: Westlund et al., 1983). PACAP-38 fibers are found scattered throughout the AP, but their density is quite low compared to that of GAP-43. However, the density and distribution of SP and CGRP in the rat AP appears quite similar to that of GAP-43.

SP and CGRP co-localization occurs primarily in peripheral sensory neurons (Hokfelt et al., 1992; Micevych and Kruger, 1992). The likelihood that some of the GAP-43 afferents in the AP contain SP and CGRP strongly suggests that these fibers could be of sensory origin. Reinforcing this possibility is the fact that collateral sprouting by sensory neurons has been shown to occur in the skin of adult rats (Diamond et al., 1992; Nixon et al., 1984), and by the trigeminal nerves into the taste buds, lower lip, chin, tongue, and teeth (Kinnman and Aldskogius, 1988; Robinson 1983). Furthermore, peripheral axotomy of primary sensory neurons has been shown to cause an increase in GAP-43 mRNA expression that is maintained for at least 30 days following injury (Chong et al., 1994), a period when these neurons are known to sprout and/or regenerate.

If the GAP-43-ir fibers are of sensory origin, the most probable source would be the trigeminal semilunar ganglia given their close proximity to the AP. This innervation would likely stem from either the meningeal rami of the maxillary and/or mandibular

branches of the trigeminal nerve. In support of this hypothesis, Ju et al (1993; 1994) have described the presence of axons immunoreactive for SP and CGRP in the rat AP whose density, like GAP-43-ir, is seen to increase after ADX. These SP/CGRP fibers appear quite similar to the GAP-43 positive processes found in the AP (unpublished data from this laboratory). Moreover, the presence of GAP-43-ir fibers in trigeminal nerves (Del Fiacco et al., 1994; Erzurumlu et al., 1989; Verze et al., 1999), along with the co-localization of GAP-43 with both SP and CGRP in human trigeminal nerve fibers (Del Fiacco et al., 1994) suggests that the GAP-43-ir processes in the AP could arise from the ganglia. In further support of this hypothesis are studies showing that a subset of the SP/CGRP fibers found in the AP and trigeminal ganglia contain PACAP-38 (Mikkelson et al., 1995; Moller et al., 1993).

Another possible source of the GAP-43 afferents found innervating the AP is the autonomic nervous system (ANS). GAP-43 protein has been found to be widespread throughout the ANS including the superior cervical ganglia (SCG), iris, and enteric system (Hou et al., 1998; Stewart et al., 1992). Moreover, GAP-43 mRNA has been reported to be up-regulated in the rat SCG after preganglionic transection (Hou et al., 1998). If the GAP-43 processes found in the AP are of either sympathetic or parasympathetic origin the potential sources would be the superior cervical ganglia (SCG) or the pterygopalatine ganglion, respectively. In support of the possibility that the GAP-43-ir innervation arises from the SCG, sympathetic fibers from the contralateral ganglion have been reported to undergo collateral sprouting into the partially denervated pineal gland after unilateral ganglionectomy (Dorney et al., 1985). Furthermore, compensatory collateral sprouting by aminergic and cholinergic autonomic afferents has

been shown to occur in the hippocampal formation (Gage et al., 1983a, Gage et al., 1983b). SP and CGRP may also be co-expressed by autonomic neuronal populations, but to a much lesser extent than sensory neurons (Hokfelt et al., 1992).

The GAP-43-ir innervation of the AP could also originate from central neuroendocrine afferents. This possibility is supported by data showing that CNS neurosecretory axons within the neural lobe of the adult rat pituitary are capable of collateral sprouting (Watt and Paden 1991, Watt et al., 1999). Furthermore, if the GAP-43-ir processes are in fact SP/CGRP positive they may arise from the periventricular region where it is known that CGRP and a small population of SP perikarya are present in the rat (Hokfelt et al., 1992; Kresse et al., 1992; Okamura et al., 1994), or other brain regions containing one or both of these peptides.

Experiments Designed to Investigate the Possible Sources of Origin of the Axonal Processes Staining Positive for GAP-43 in the AP

Four experiments were completed. The first study involved post-mortem tract tracing of the trigeminal nerve (CN V) using the fluorescent dialkylcarbocyanine lipophilic anterograde tracer DiI. This experiment was done to test the hypothesis that the GAP-43-ir AP processes are sensory in origin. As described previously, the presence of AP afferents immunoreactive for both SP and CGRP strongly suggests that the GAP-43-ir processes could be primary sensory neurons extending from the maxillary and/or mandibular branches of the trigeminal nerve.

The second experiment involved the bilateral removal of the superior cervical ganglia (SCGX) to test the hypothesis that the GAP-43-ir processes in the rat AP are of

sympathetic origin. The existence of a peripheral sympathetic projection to the hypothalamic-pituitary axis, derived from the SCG, makes the ganglia another likely source of origin of the GAP-43-ir processes found in the rat AP. If the fibers are sympathetic in origin, the complete ablation of SCG afferents to the AP should lead to a reduction in GAP-43-ir processes.

The last two studies investigated the possibility that the direct innervation of corticotrophs by GAP-43-ir processes could be of central origin. In order to test this hypothesis retrograde tracing from the AP was performed using both DiI and Fluorogold (FG). Because of the fast axoplasmic retrograde transport of the FG it was possible to examine SCG and trigeminal ganglia as an additional means of investigating the potential innervation of the AP by sympathetic and/or sensory neurons.

MATERIALS AND METHODS

Experimental Design

These experiments have been designed to investigate the possible source(s) of processes immunoreactive for the growth associated protein GAP-43 in the anterior lobe of the pituitary of the adult male Holtzman rat. The experiments are an extension of work previously done in this laboratory showing that GAP-43 axon-like projections sprout to specifically and thoroughly contact corticotrophic cells during a period in which these cells are known to be undergoing both hypertrophy and hyperplasia in response to adrenalectomy (Paden et al., 1994a; Paden et al., 1998). The goal of this project is to determine the source of GAP-43-ir innervation in the rat AP by completing four studies: (1) post-mortem dialkylcarbocyanine (DiI) anterograde axonal tracing of the trigeminal nerves (CN V), (2) effects of superior cervical sympathetic ganglionectomy on GAP-43-ir, (3) post-mortem DiI retrograde axonal tracing from the AP, and (4) fluorogold retrograde axonal tracing from the AP in the living animal. The studies involving the DiI tracing were done as a collaborative effort with Dang Khoa Duong. Dr. Janee Gelineau-van Waes, DVM, served as a surgical consultant in exposing the AP, and Dr. Mark Taper assisted with the nonparametric statistical analysis.

The animals used in all of these experiments were adult male Holtzman albino rats between 4 and 6 months of age. These rats were either bred or ordered from Harlan

stocks, and kept at the Montana State University Animal Resource Center, an American Association for Accreditation of Laboratory Animal Care accredited facility. All applicable guidelines from the NRC Guide for the Care and Use of Laboratory Animals were followed throughout these experiments, and all protocols were approved by the Montana State University Institutional Animal Care and Use Committee.

I. Dialkylcarbocyanine (DiI) Anterograde Tracing of the Trigeminal Nerve (CN V)

Other studies have shown that DiI uniformly labels neurons via lateral diffusion through the lipophilic plasma membrane of axons at a rate of about 0.2-0.6 mm per day in fixed specimens (Balice-Gordon et al., 1993; Godement et al., 1987). The diffusion rate is doubled at approximately 40^{o} C (Molecular Probes, USA). The diunsaturated alkyl substituents found on Fast DiI result in an accelerated rate of diffusion within fixed membranes at a rate of approximately 1.0-1.2 mm a day (Molecular Probes, USA). In general, the DiI tracers do not transfer from labeled to unlabeled cells unless the membrane is disrupted. Furthermore, DiI has been shown to be very stable, with some studies reporting that the diffusion of the dye can be followed up to two years in aldehyde-fixed tissue (Vidal-Sanz et al., 1988; von Bartheld et al., 1990).

Animals and Tissue Preparation

Adult male Holtzman rats weighing between 300 and 500 grams were kept under a 12:12 hour light:dark cycle and given access to food and water *ad libitum*. Intact animals were sacrificed by transcardiac perfusion under Metofane anaesthesia using a modified Nakane's fixative (Paden et al., 1994) consisting of 2.8% paraformaldehyde in 0.012 M phosphate buffer with 0.106 M lysine and 0.014 M sodium meta-periodate. The perfused rats were decapitated, and their heads were post-fixed in 4% paraformaldehyde overnight. A careful, caudal micro-dissection through the occipital bone, ventral to the cerebellum, was done to expose the semilunar ganglia just rostral to the pons. The trigeminal nerves were bilaterally sectioned at the point where they left the caudal end of their respective semilunar ganglia using a dissecting microscope and an eye-blade micro-scalpel. After blotting the cut trigeminals with filter paper to partially dehydrate the free nerve endings, the dialkylcarbocyanine axonal tracer DiI was applied to the transected nerves.

Preparation of DiI for Anterograde Labeling

Regular DiIC_{18} crystals (Molecular Probes) were crushed into fine dust using a mortar and pestle. Ten mg of DiIC_{18} particulate were dissolved into 25 mg of silicon grease (Thomas Scientific, "Lubriseal") and 75 µl of absolute ethanol while stirring vigorously. The reconstituted DiI formed a paste and was stored at -20^{o} C until it was used. Due to difficulties in getting the DiI paste to stick to the cut trigeminal nerve

endings other methods of DiI application were also investigated. The other methods for delivering regular $DiIC_{18}$ were to dissolve 10 mg of the dye particulate in either 25 µl of sesame oil, or in 100 µl of absolute methanol immediately before application; $DiIC_{18}$ crystals were also inserted directly into the transected nerve endings. Use of absolute methanol, sesame oil, or direct application of DiI crystals seemed to work the best, possibly because more DiI stuck to the cut nerve ending when these methods were employed.

Fast DiI (Molecular Probes), which differs from regular DiI by having diunsaturated 9,12-C_{18} alkyl substituents in place of the saturated C_{18} tail of regular $DiIC_{18}$, was also utilized due to its reported accelerated rate of diffusion within lipophilic membranes (Vidal-Sanz et al., 1988; von Bartheld et al., 1990). Approximately 10 mg of Fast DiI was either dissolved in 25 µl of sesame oil, 100 µl of absolute methanol, or inserted into the cut nerve in its native crystalline form.

Application of DiI to Transected Trigeminal Nerves

A very fine paintbrush, trimmed to have only a few hairs, was used to apply the regular or Fast DiI paste to the cut trigeminal nerves, or an eye-blade micro-scalpel was used to punch DiI crystals into the cut nerves. Because DiI is soluble in alcohol, a small drop of 70% ethanol was applied to the cut nerves after DiI application in an effort to get the DiI equally distributed over the entire surface. The tissue was then allowed to dry for 5 min in order to assure the crystals or paste adhered to the nerves. Next the rat heads were placed dorsal-side down into 4% paraformaldehyde and sealed in Nalgene jars in

order to keep the tissue from dehydrating during the long incubation period. The rat heads were then placed into a 40° C oven to help facilitate dye transport and incubated for 2 to 6 months.

Tissue Analysis

Pituitaries, trigeminal semilunar ganglia, and trigeminal nerve branches from their ganglion to the point where they entered into their respective foramina were removed and washed in 0.01M phosphate-buffered saline (PBS) overnight. The trigeminals and pituitaries were then embedded in molds filled with a 2:1 mixture of egg yolk and 12% gelatin, and placed in 2% paraformaldehyde at 4°C for 48 h. Forty μm coronal sections were cut using a Vibratome, rinsed twice in PBS, mounted on gelatin-chrome alum subbed slides, and coverslipped using Mowiol (Hoechst, purchased from Aldrich Chemical Company) lacking glycerin (Aquamount, Permafluor, glycerin and Vectashield were found to cause diffusion of the DiI).

Both Regular $DiIC_{18}$ and Fast DiI are optimally excited at 549 nm, with fluorescent emission at approximately 565 nm. Therefore, rhodamine epifluorescent microscopy was used to visualize DiI labeling in sections of trigeminals and pituitaries; and to determine the distance of dye travel in the trigeminals, along with its localization in the anterior pituitary. The filter block on the Nikon epifluorescence microscope consisted of an excitation filter passing wavelengths of 535~550 nm (main wavelength 546 nm), a DM 580 dichroic mirror, and a 580W eyepiece-side barrier cutoff filter.

II. SCGX Experiment

Animals and Tissue Preparation

Adult male Holtzman rats weighing between 250 and 300 grams were kept under a 12:12 hour light:dark cycle and were given access to food and water *ad libitum*. Bilateral SCGX or the corresponding sham operation were performed under general anaesthesia induced with a mixture of 26 mg ketamine plus 5 mg xylazine plus 0.8 mg acepromazine per kg of body weight (im). The salivary glands were exposed through a ventral incision in the neck. Retraction of the salivary glands and underlying strap muscles exposed the SCG dorsal-medial to the bifurcation of the common carotid artery into its internal and external branches. The ganglia were completely removed from both sides; sham operations included all manipulations except for SCG removal. Bilateral palpebral ptosis, as defined by Cardinali et al (1986), in conjunction with histological analysis of the removed tissue (Figure 5) were used as criteria for determining the success of the SCGX. Animals not exhibiting bilateral palpebral ptosis, and therefore having received an incomplete SCGX, were discarded. SCGX rats and SCGX-sham rats (n=4 per group) were sacrificed at 2 and 8 days after surgery by transcardiac perfusions under Metofane anaesthesia using a modified Nakane's fixative (Paden et al., 1994). Pituitaries were removed, immersion fixed for an additional 24 h, and then washed in 0.01M phosphate-buffered saline (PBS) overnight. The pituitaries were then embedded in molds filled with a 2:1 mixture of egg yolk and 12% gelatin and placed in 2%

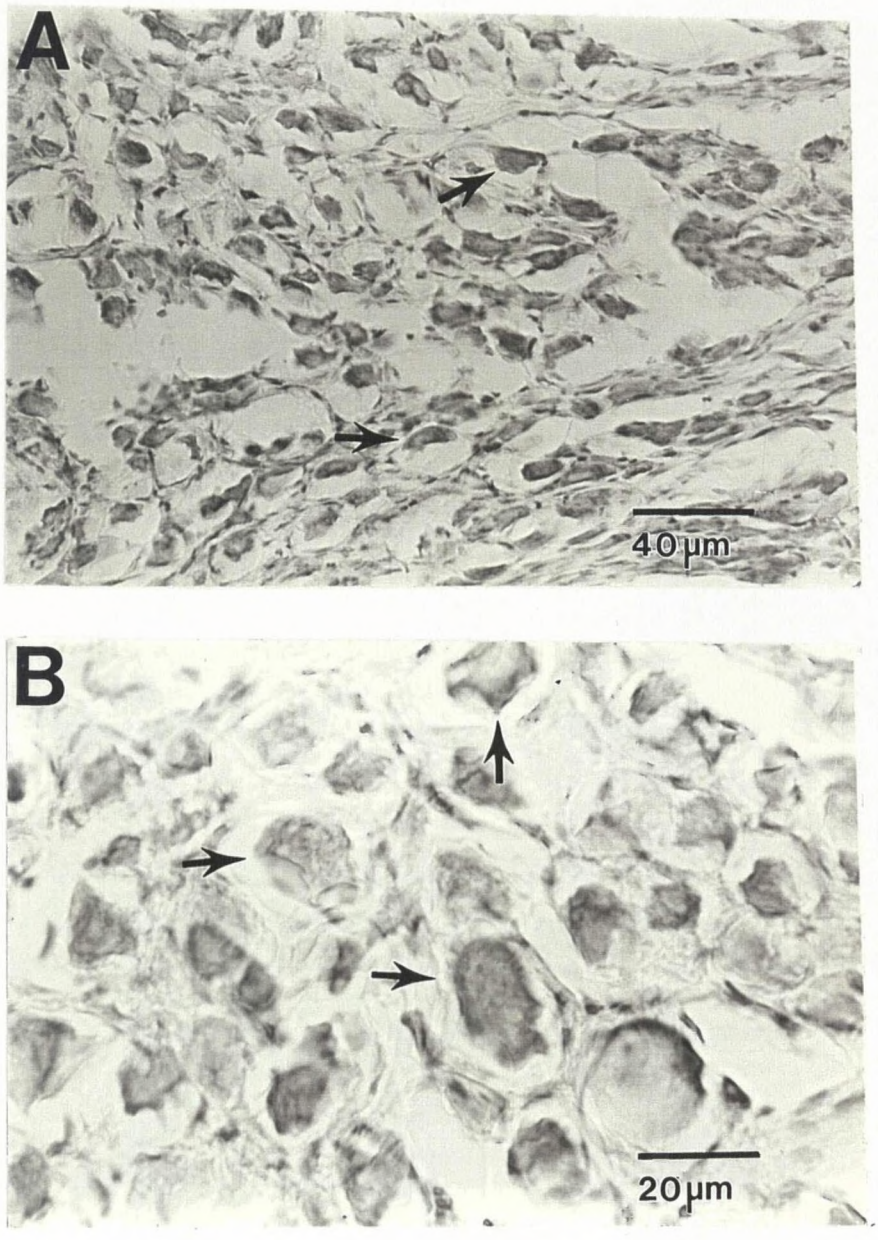

Figure 5. Light micrographs of 15 μm cryocut sections through the superior cervical ganglia (SCG) of the rat, stained with cresyl violet. Both SCG from each animal undergoing SCGX were analyzed to confirm that bilateral SCGX was successful. Neural cell bodies (arrows) were seen at both 400X **(A)** and 800X **(B)**.

paraformaldehyde at 4°C for 48 h. Forty μm coronal sections were cut using a Vibratome, rinsed twice in PBS, and stored in a cryoprotectant [300 g sucrose + 10 g polvinyl pyrolidone (Sigma PVP40) dissolved in a mixture of 500 ml 0.1 M phosphate buffer and 300 ml ethylene glycol] at -20° C until used.

A fifth group of animals received bilateral adrenalectomies (ADX) 4 days after successful SCGX under general anaesthesia as described above. Following surgery, rats were maintained on 0.9% saline solution and lab chow *ad libitum*. SCGX+ADX rats (n=4) were sacrificed 4 days after ADX and pituitaries were prepared for analysis as described above for SCGX animals.

Immunocytochemistry

All steps were performed at room temperature unless otherwise noted. Sections were first rinsed 2 x 10 min in 0.05 M tris buffered saline (TBS) to remove cryoprotectant. Sections were then pretreated with 0.1% H_2O_2 in methanol for 10 min to eliminate endogenous peroxidase activity and rinsed 2 x 10 min in TBS, followed by a 1 h incubation in a blocking buffer consisting of 10% normal horse serum (NHS) in TBS. Sections were then placed directly into mouse monoclonal anti-GAP-43 IgG (Boehringer Mannheim) at a 1:5000 dilution in an incubation buffer consisting of 2% bovine serum albumin (BSA) and 5% NHS made in TBS containing 1.8% NaCl and 0.2% Tween-20 (TBS_2T), and were agitated on a rocker at 4° C for 48 h. Following a single rinse in TBS_2T, sections were washed in incubation buffer overnight on a rocker at 4° C, rinsed in TBS_2T, and incubated in biotinylated horse anti-mouse IgG (Jackson Labs, 1:200) in

incubation buffer for 2 h. Sections were then rinsed 2 x 10 min in TBS_2T, then 2 x 10 min in TBS, and placed in avidin-biotin complex (ABC) (Vector *elite*) in TBS for 90 min. Following 3 x 10 min rinses in TBS, localization of bound peroxidase was done using a solution of 0.5 mg/ml diaminobenzidine (Sigma), 8 mg NH_4Cl, and 40 mg glucose in 19.5 ml TBS, with generation of H_2O_2 using glucose oxidase (Sigma type VII). After final 3 x 10 min rinses in TBS, sections were mounted on gelatin-chrome alum subbed slides, dehydrated in a graded ethanol series, cleared in xylene, and cover slipped with Permount.

Tissue and Statistical Analysis

GAP-43 staining was examined using 10x, 20x, and 40x objectives on a Nikon Optiphot-2 light microscope. Black and white photos of the staining were taken with a Nikon FX-35WA camera mounted onto the scope.

Two sections from each animal were coded for blind analysis, then ranked independently by 3 observers according to the density of GAP-43-ir. The three ranks for each animal were averaged to give an over-all rank of axonal density for the 2-day sham, 2-day SCGX, 8-day sham, 8-day SCGX, and SCGX+ADX groups of animals. This analysis was replicated with the over-all ranks from the two trials averaged to give an ordinal rank for each animal. Nonparametric analysis was then performed using the Kruskal-Wallis test statistic (Conover, 1980). A chi-square distribution was used, and the null hypothesis rejected at the 0.05 level. Post-hoc multiple comparisons were done

upon rejection of the null hypothesis to determine which pairs of populations were different.

III. Dialkylcarbocyanine (DiI) Retrograde Tracing from the Anterior Pituitary

Animal and Tissue Preparation

Adult male Holtzman rats weighing between 300 and 500 grams were kept under a 12:12 hour light:dark cycle and given access to food and water *ad libitum*. Bilateral adrenalectomies (ADX) were performed under general anaesthesia as described above. Following ADX, rats were maintained on 0.9% saline solution and lab chow *ad libitum*. The 0.9% saline drink was necessary in order to replace adrenal mineralocorticoids. Adrenalectomized rats (n=5 per group) were sacrificed at 4, 7, and 10 days after surgery, along with intact controls (n=3 per group), by transcardiac perfusions as described above. The perfused rats were decapitated, and heads were post-fixed in 4% paraformaldehyde overnight. A careful caudal micro-dissection through the sphenoid bone, ventral to the pituitary, was done to expose the anterior lobe. With the aide of a dissecting microscope an eye-blade micro-scalpel was used to make a shallow coronal incision in the ventral-caudal surface of the AP where small regular DiIC_{18} or Fast DiI crystals were inserted to fill the incision. The rat head was then placed ventral-side up into a Nalgene jar filled approximately 1/2" with 4% paraformaldehyde and then incubated in a 40^{o} C oven for 2 to 8 months to allow for dye diffusion along the lipid bilayer membrane of the axons innervating the AP. The heads were kept in a humid environment rather than submerged

in 4% paraformaldehyde in an attempt to reduce dye diffusion into surrounding membranes.

In an effort to reduce diffusion of the DiI into the neural lobe, post-mortem neurohypophysectomies were done in another population of 4-day ADX (n=5 per group) and intact (n=3 per group) animals. The same methodology as described above was used to gain exposure of the pituitary. Next, an eye-blade micro-scalpel and a slightly curved set of micro-dissecting forceps were used to excise the neural lobe. Extra caution was taken during this dissection so as to leave the pituitary stalk intact. DiI application and tissue preparation was done in a manner similar to that described for retrograde tracing of the non-neurohypophysectomized animals as explained above.

Tissue Analysis

At various time points between 2 and 8 months after DiI application brains were removed, and then washed in 0.01M PBS overnight. The pituitaries and brains were then embedded in molds filled with a 2:1 mixture of egg yolk and 12% gelatin and placed in 4% paraformaldehyde at 4^{o}C for 48 h. Forty μm coronal sections were cut using a Vibratome, rinsed twice in PBS, mounted on gelatin-chrome alum subbed slides, and coverslipped using Mowiol lacking glycerin. DiI labeling of brain and pituitary sections was analyzed using rhodamine epifluorescent microscopy as previously described.

IV. Fluorogold Retrograde Tracing from the Anterior Pituitary

Surgical Procedures

Adult male Holtzman rats weighing between 300 and 500 grams were kept under a 12:12 hour light:dark cycle and given access to food and water *ad libitum*. Bilateral adrenalectomies (ADX) were performed under general anaesthesia as described above. Following ADX, rats were maintained on 0.9% saline solution and lab chow *ad libitum*. Adrenalectomized rats were housed for 4 days to permit sprouting of GAP-43 positive presumptive axons to occur (Paden et al., 1998). Iontophoresis of FG was then performed into the ventral AP of intact and ADX rats.

To expose the anterior pituitary rats were anaesthetized with the ketamine cocktail. A midline incision was made on the ventral surface of the neck, directly over the esophagus. Bilateral retraction of the glandula mandibularis was achieved to expose the larynx and glandula thyreoidea. The right strap muscle was retracted, and the esophagus and trachea were gently elevated with a periosteal elevator. A modified bone chisel (a Freer beveled to a width of 5 mm) was used to detach musculature from the base of the skull to expose the sphenoid bone. A dental burr was used to drill through the sphenoid bone just rostral to the pituitary trigone exposing the ventral AP. During the transphenoidal craniotomy Bone Wax and Gel Foam (Upjohn) were used to stop the bleeding of the sphenoid spongy bone. Next a #11 scalpel blade was used to remove the dural covering of the pituitary.

After exposure of the AP, a stereotaxic device was used to lower a 20 μm glass micro pipette filled with 1% Fluorogold in 0.9% saline vehicle into the AP. After micro pipette placement a +5 μA alternating current (7 seconds on, 7 seconds off) was passed through the micro pipette for 20 min using a Stoelting constant current source to iontophoretically deliver the FG as described by Canteras et al (1992). After tracer delivery the Stoelting constant current source was put on standby, and the electrode kept in place for 5 min in an attempt to try and reduce diffusion of the FG up the electrode tract. The electrode was retracted and then moved a millimeter laterally and the process was repeated for another 20 min. During the delivery of FG the exposed tissue was bathed in normal saline to prevent dehydration. Following FG deliver a small piece of subcutaneous fat was excised and placed over the exposed AP, and the retracted tissue returned to its original position. The skin was closed with 3.0 braided silk sutures. The intact animal was allowed to survive 2 days; while the ADX animals were sacrificed at 2, 7, and 14 days after FG iontophoresis.

Tissue Preparation

Rats were sacrificed by transcardiac perfusion using a modified Nakane's fixative under Metofane anaesthesia (Paden et al., 1994). Brains, pituitaries, trigeminal ganglia, and superior cervical ganglia (SCG) were removed, immersion fixed for an additional 24 h, and then washed in 0.01M PBS overnight. Then all tissue specimens, except the SCGs, were embedded in molds filled with a 2:1 mixture of egg yolk and 12% gelatin and placed in 2% paraformaldehyde at 4^{O}C for 48 h. Forty μm coronal sections were cut

using a Vibratome, rinsed twice in PBS, mounted on gelatin-chrome alum subbed slides, and coverslipped using Vectashield (Vector Laboratories). The SCGs were incubated overnight in a 30% sucrose solution at 4^{o} C, then embedded in OCT compound on a cryostat chuck. Fifteen μm SCG sections were cut using a cryostat, mounted on gelatin-chrome alum subbed slides, and coverslipped using Vectashield.

FG is optimally excited at 350-395 nm, with fluorescent emission at approximately 530-600 nm. Therefore, UV epifluorescent microscopy was used to visualize FG in sections of brains, pituitaries, SCGs, and trigeminal nerves/ganglia. The Nikon epifluorescence microscope was equipped with a wide band ultraviolet (UV10 filter block) excitation filter passing wavelengths of 330~380 nm (main wavelength 365 nm), a DM 400 dichroic mirror cutoff, and a 420 nm eyepiece-side barrier cutoff filter. A Nikon FDX-35 model H-III camera mounted on the microscope was used to photograph the neuronal populations labeled by the FG.

RESULTS

Post-Mortem DiI Anterograde Tracing of the Trigeminal Nerves

In an effort to determine if the GAP-43-ir fibers found in the anterior lobe of the pituitary are of sensory origin anterograde tracing of the trigeminal nerves was achieved using the long-chain dialkylcarbocyanine axonal tracer, DiI. A total of 10 animals that had either regular DiI or Fast DiI applied to the transected nerve endings were sacrificed by transcardiac perfusion 2, 3, 4, 5, and 6 months after DiI application. Tissue was sectioned and visualized using rhodamine epifluorescent microscopy. Both the regular DiI and Fast DiI appeared to have appropriately labeled the transected trigeminal nerve fibers via longitudinal diffusion within the axonal plasma membrane. The labeling appeared as intense red fluorescing membranes encompassing individual axons in transverse sections within all of the nerves analyzed. The longitudinal sections of the nerves revealed extensive fibers running in parallel to one another. The fibers coursed the length of the dissected trigeminals, from where the nerves were transected to the various points at which the branches delve into their respective foramina.

Fluorescent microscopy revealed very little staining in coronal sections of the anterior lobe. No axons were observed in the parenchyma of the AP in any of the ten animals who had their trigeminals labeled with the DiI. In many of the tissue sections there appeared to be diffusion of the DiI into all of the lobes of the pituitary gland. This

staining appeared as a diffuse bright red glow with no definitive structures seen. However, in two of the animals there appeared to be very rare fibers running along the periphery of the anterior lobe. These fibers appeared to be coursing through the hypophysial fascia in very close proximity to the AP, with a few apparently non-related processes seen within the actual AP parenchyma. It was not possible to determine whether or not any of the fibers seen in the fascia entered the AP at some point, leaving open the possibility that a few axons do in fact branch off of the trigeminal nerve to innervate the AP from the periphery.

Unfortunately, too long an interval was allowed to pass before attempting to take pictures of these sections. The loss of specific labeling of the DiI occurred quite rapidly, and was likely due to the transfer of the dye to the extracellular matrix when the neuronal cell membranes were disrupted during the sectioning process. This has been reported to have occurred in several instances in other laboratories that have used DiI in a similar manner (Honig and Hume, 1986; Thanos, 1991).

Effects of SCGX on GAP-43-ir in the Anterior Pituitary

To determine if the GAP-43-ir fibers are of sympathetic origin immunocytochemical staining techniques were utilized to examine the distribution and response of GAP-43-ir processes in the rat AP following bilateral removal of the superior cervical ganglia (SCGX). In the initial experiment the effects of SCGX on the response of GAP-43-ir axons to subsequent bilateral ADX were examined. It has previously been shown that ADX induces a dramatic increase in the extent and intensity of GAP-43-ir (Lu

et al., 1995) that were subsequently interpreted as collateral sprouting of GAP-43-ir axons that target corticotrophs in the rat AP (Paden et al., 1998). It was therefore reasoned that this response should be abolished by prior SCGX if these axons arise from the SCG. Rats underwent ADX 4 days following SCGX and were sacrificed after an additional 4 days (SCGX + ADX). A dramatic increase in the density of GAP-43-ir axons in the AP was apparent in the SCGX + ADX subjects compared to rats undergoing SCGX alone (Figure 6).

Furthermore, the increase in intensity of axonal staining in the AP of SCGX + ADX animals was similar to that observed in the AP of rats undergoing ADX only (Figure 6). The increased intensity of GAP-43-ir seen in both ADX and SCGX + ADX groups was present throughout the AP, and was manifested primarily as an increase in the number of punctate clusters seen surrounding gland cells and as a greater number of fine beaded individual axonal-like processes coursing through the lobe.

In the second experiment we compared the extent of axonal staining for GAP-43 in the AP at both 2 and 8 days after bilateral SCGX (n=4 per time point) or sham surgery (n=3 per time point). No reduction in axonal density was apparent in the AP of any of the 8 day SCGX animals compared to 8 day sham-operated (Figure 7), consistent with the conclusion that GAP-43-ir processes were not severed by SCGX. However, a transient increase in density of GAP-43-ir axons was apparent in the AP of both the 2 day SCGX and the 2 day sham-operated groups (Figure 7), suggesting that the stress associated with anesthesia and surgery caused an increase in GAP-43 protein in the axonal-like processes present in the AP.

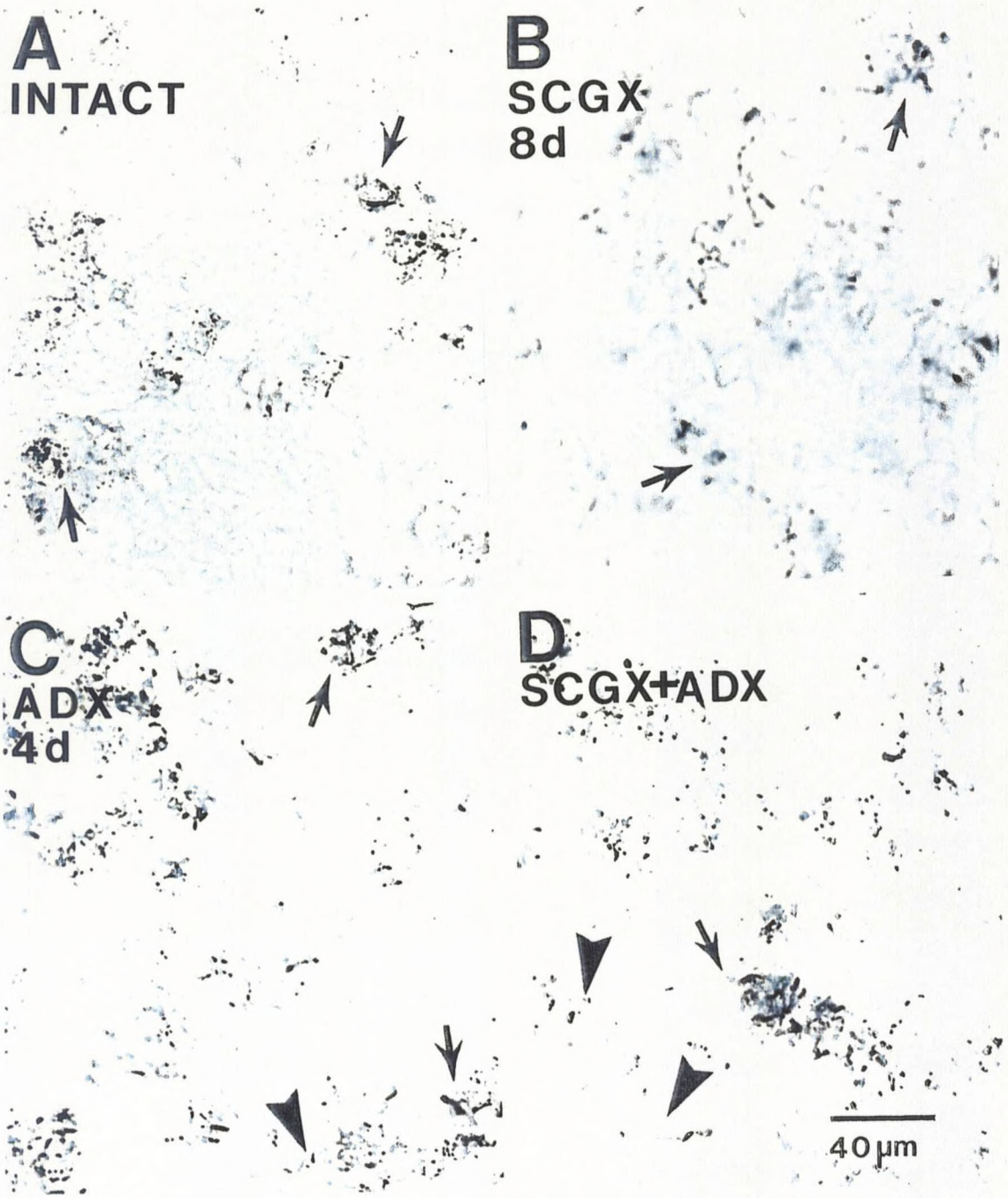

Figure 6. The fact that staining is not reduced 8 days following SCGX **(B)** compared to intact animals **(A)** demonstrates that the GAP-43-ir axons do not originate in the SCG. This conclusion is supported by the increase in staining that was observed when ADX was performed 4 days after SCGX **(D)**. This up-regulation of GAP-43-ir processes in the SCGX+ADX **(D)** rat appears equivalent to that seen in the ADX rat **(C)**. Both fine beaded axons (arrowheads) and punctate clusters of putative axon terminals (arrows) are visible, but appear especially dense in 4 day post-ADX and SCGX+ADX animals. Magnification is 400X.

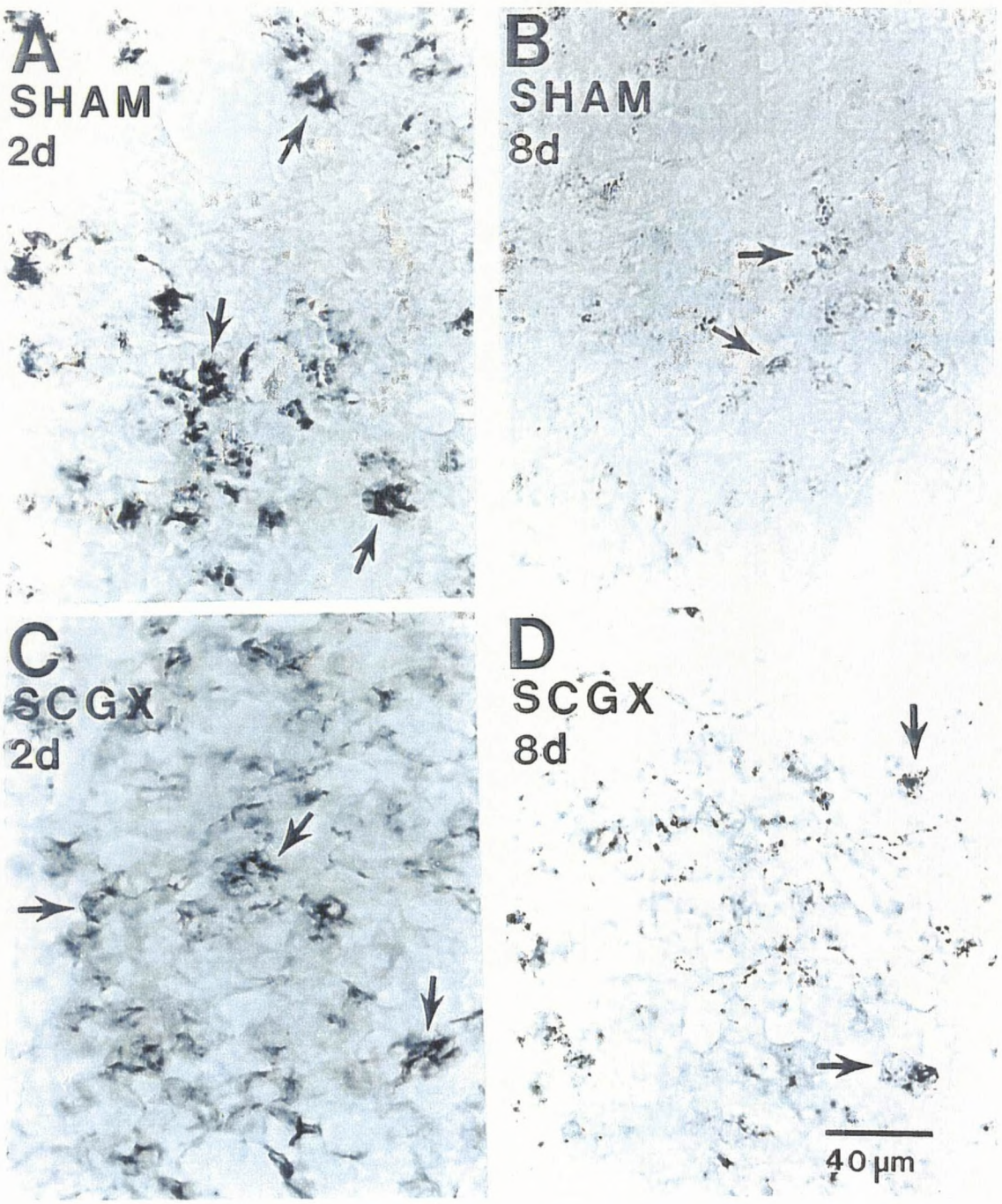

Figure 7. A substantial increase in the number of GAP-43-ir processes was apparent 2 days after SCGX or sham operation **(A, C)** compared to 8 day post-SCGX and post-sham groups **(B, D)**. Furthermore, no significant difference was seen between the two 2 day groups or the two 8 day groups. The punctate staining of putative axon terminals also appeared more robust in the 2 day operated groups (arrows **A, C**) compared to that seen in the 8 day operated groups (arrows **B, D**). Magnification is 400X.

GAP-43-ir appeared as numerous fine beaded processes coursing individually or forming plexiform networks throughout the parenchyma in both SCGX and sham-operated animals, and also appeared as intensely labeled punctate structures surrounding either individual gland cells or groups of cells. These patterns were most visible in the 2 day SCGX and sham-operated groups where the intensity of staining was increased over that of the intact controls or 8 day operated groups.

The significance of apparent differences in the extent of axonal staining between experimental groups was verified using a nonparametric statistical test. Following blind ranking of two tissue sections from each rat by three independent observers based on the density of GAP-43 axonal staining, the Kruskal-Wallis test statistic was applied in conjunction with post-hoc multiple comparison tests (Conover, 1980). Results of this procedure confirmed that axonal density was not reduced 8 days after SCGX compared to sham-operated controls, and further confirmed that ADX performed after SCGX caused a significant increase in GAP-43-ir density within the AP (Figure 8). In addition, the transient increase in staining density observed in both SCGX and sham-operated control groups at 2 days post-surgery was found to be significant when compared to the 8 day SCGX and sham-operated control groups, but this increase was found to be less than that induced by ADX.

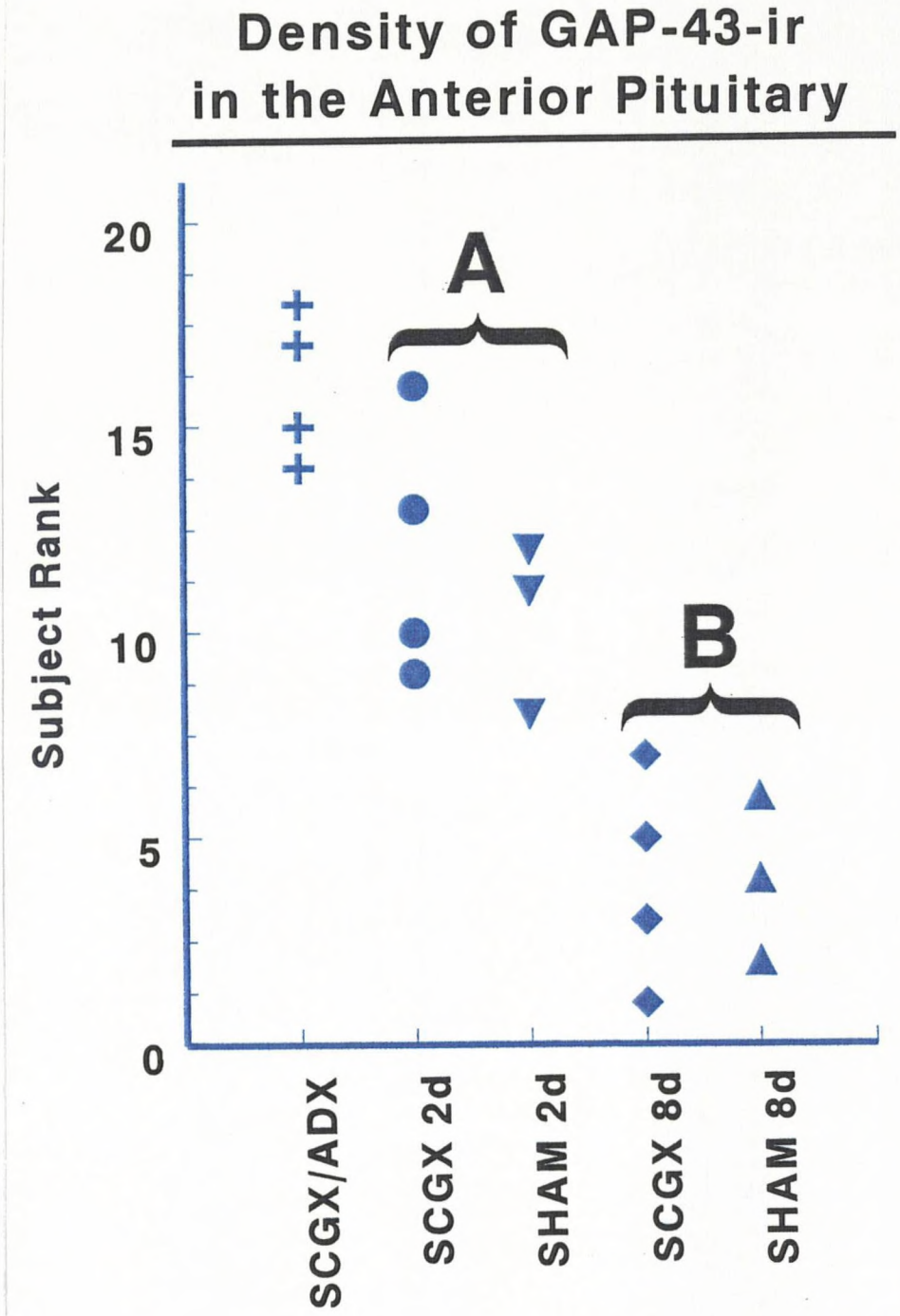

Figure 8. Nonparametric statistical analysis confirmed that the density of GAP-43-ir processes was significantly different between the SCGX+ADX, combined 2 day post-surgery **(A)**, and combined 8 day post-surgery **(B)** groups. No significant difference existed, however, between the 2 day SCGX and 2 day sham groups, or the 8 day SCGX and 8 day sham groups.

Post-Mortem DiI Retrograde Tracing from the Anterior Pituitary

In the initial DiI retrograde tracing experiment 3 intact controls and 15 ADX rats were examined. Retrograde tracing of the AP was achieved using the long-chain dialkylcarbocyanine axonal tracer, DiI. Brains were harvested and sectioned 2 to 8 months after the DiI application. Visual analysis of the pituitary gland showed a very intense red labeling at the site of the incision where the DiI was applied. However, due to diffusion of the DiI, the entire pituitary gland had a bright pink hue.

Intense labeling of axons and neural cell bodies was present throughout the hypothalamic region. In all but a few of the intact and ADX brains the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the magnocellular neurosecretory system contained intensely labeled neurons, as did the arcuate nuclei and periventricular nuclei. Labeling was also present throughout the median eminence, fornix, optic chiasm, infundibular stalk, and mammillothalamic tract; but individual axons could not be distinguished in those locations. DiI labeled axons were seen coursing throughout the hypothalamus, whereas somal staining of neurons was restricted to the magnocellular and parvocellular hypothalamic nuclei. The somal labeling appeared as bright red fluorescing membranes surrounding the neural cell bodies with axonal and dendritic processes extending off in various directions. In animal ADX 13 faint fibers were seen extending from the hypothalamic region up to the edge of the medial habenular nuclei (MHb) just lateral to the third ventricle. However, no labeled cell bodies were seen within the actual MHb. Both Fast DiI and regular DiI gave the same type of labeling.

The pink hue of the entire pituitary gland and the labeling of the magnocellular neurosecretory system indicated that the dye was diffusing out of the AP at a concentration sufficient to label axons projecting to the NL. Therefore, it was decided to try retrograde tracing from the AP with DiI in neurohypophysectomized (NHX) intact and ADX animals in an attempt to alleviate this problem. Brains from these animals were removed and sectioned 2 to 8 months after DiI application. Again, the entire pituitary, minus the neural lobe, had a pink hue with intense red labeling appearing at the site of DiI application. Rhodamine epifluorescent microscopy revealed labeling identical to that seen in the non-NHX animals. Only a few animals had no labeling, and that was likely due to disruption of the pituitary stalk upon exposure of the AP or during the NHX procedure, or while the DiI was being applied. The staining was the same using either the Fast DiI or regular DiI with removal of the neural lobe having minimal effects on the intense labeling found present within the magnocellular neurosecretory system. In two of the animals (ADX-NHX #6, and ADX-NHX #10) faint axons were seen extending up to the MHb, similar to that observed in the ADX #13 animal. As was the case in the DiI anterograde tracing study, too long an interval was allowed to pass before attempting to photograph the sections, resulting in diffusion of the DiI out of the cell bodies and axons.

Fluorogold Retrograde Tracing from the Anterior Pituitary

Fluorogold (FG) retrograde tracing was done to examine all possible sources of neural innervation into the rat AP. FG was iontophoresed into the AP of a living intact rat that was allowed to survive an additional 2 days, as well as into the AP of 4-day post-

ADX animals (n=1 per time point) that were then allowed to survive an additional 2, 7, or 14 days. The survival periods were chosen to provide enough time for the dye to undergo endocytosis by the AP axons and retrograde transport to neuronal cell bodies regardless of their distance from the AP. Pituitary glands, brains, trigeminal nerves and attached semilunar ganglia, and the superior cervical ganglia (SCG) were examined in this experiment.

Pituitary glands were sectioned in the coronal plane to confirm the site of FG injection. All 4 rats, except the 7-day ADX animal, had two overlapping injection sites just off the mid-line about 2 mm deep that formed a sphere approximately 1.5 mm in diameter (Figure 9). Staining appeared as a bright gold field with a well-defined boundary, standing out quite dramatically from the blue-purple hue of the tissue. In all but the 7-day ADX animal the FG appeared to diffuse throughout the parenchyma, with no labeling of cells present. Analysis of the 7-day ADX animal revealed the FG injection to be very shallow within the AP, with most of the labeling confined to the superficial hypophysial fascia covering the gland (Figure 9D). FG in the 7-day ADX animal appeared to be labeling fibroblasts within the fascia (Figure 9D, arrowheads). There was no detectable diffusion of the FG into the intermediate lobe or neural lobe of the pituitary in any subject (Figure 10).

Analysis of the SCG was done as an additional means of determining if axons in the AP are of sympathetic origin. However, no FG labeling of axons or somata was observed in cryosections of the SCG of any subject (Figure 11). All that was visible in these sections was the faint light-blue autofluorescence of occasional cells against the darker blue-purple background.

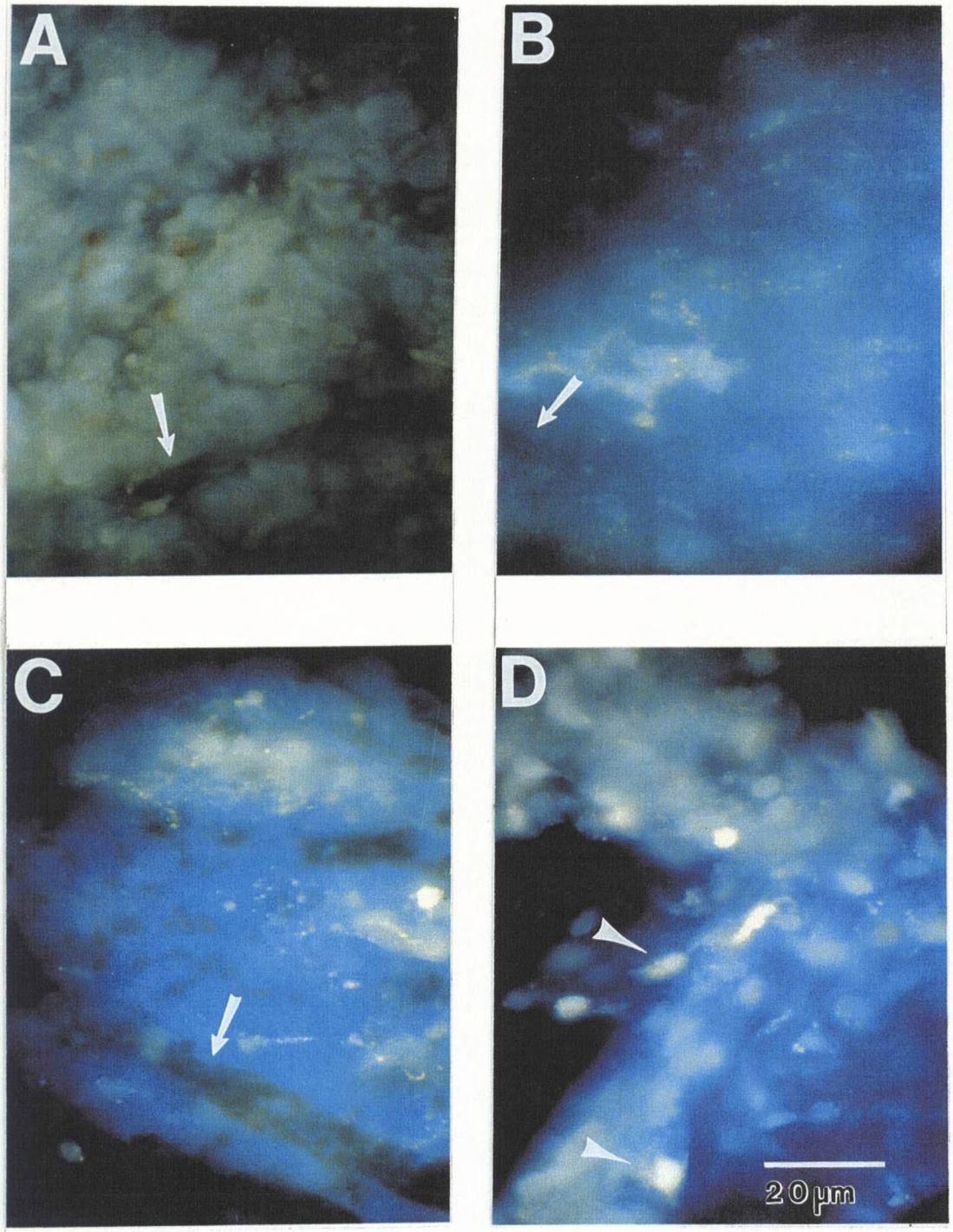

Figure 9. Micrographs confirming successful iontophoresis of FG into the rat AP. **(A)** 14 day FG-ADX, **(B)** 2 day FG-ADX, **(C)** 2 day FG-Intact, **(D)** 7 day FG-ADX rats. The micro-electrode tract can be seen in micrographs **A, B** and **C** (arrows). The punctate cellular staining (arrowheads) seen in **D** appears to be specific to fibroblasts in the superficial hypophysial fascia covering the AP. Magnification is 900X.

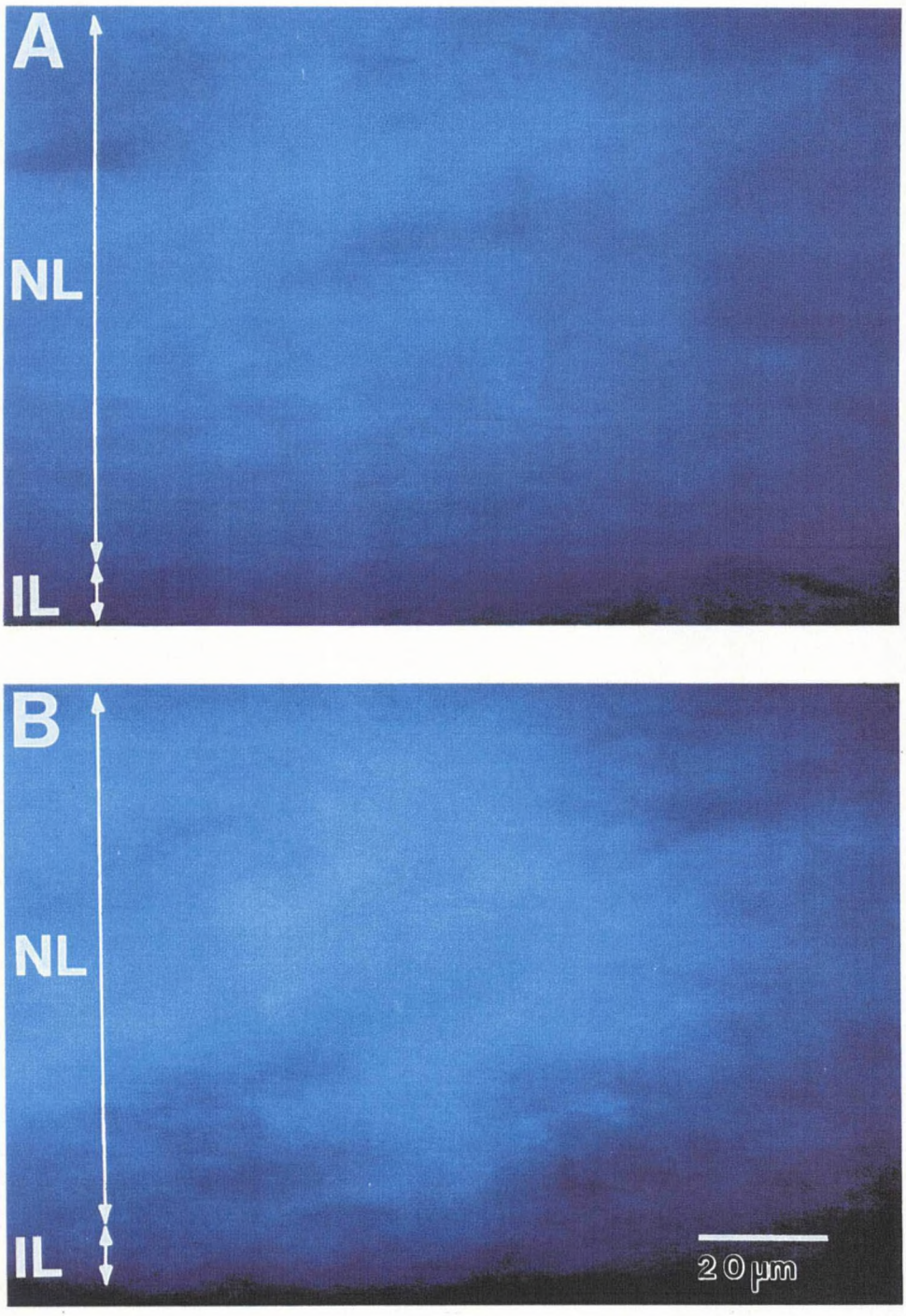

Figure 10. Representative UV epifluorescent micrographs of the intermediate (IL) and neural (NL) lobes of pituitaries following iontophoresis of FG into the AP. Unlike the DiI study, there was no diffusion of the FG into the intermediate lobe or neural lobe in any subject. Magnification is 900X.

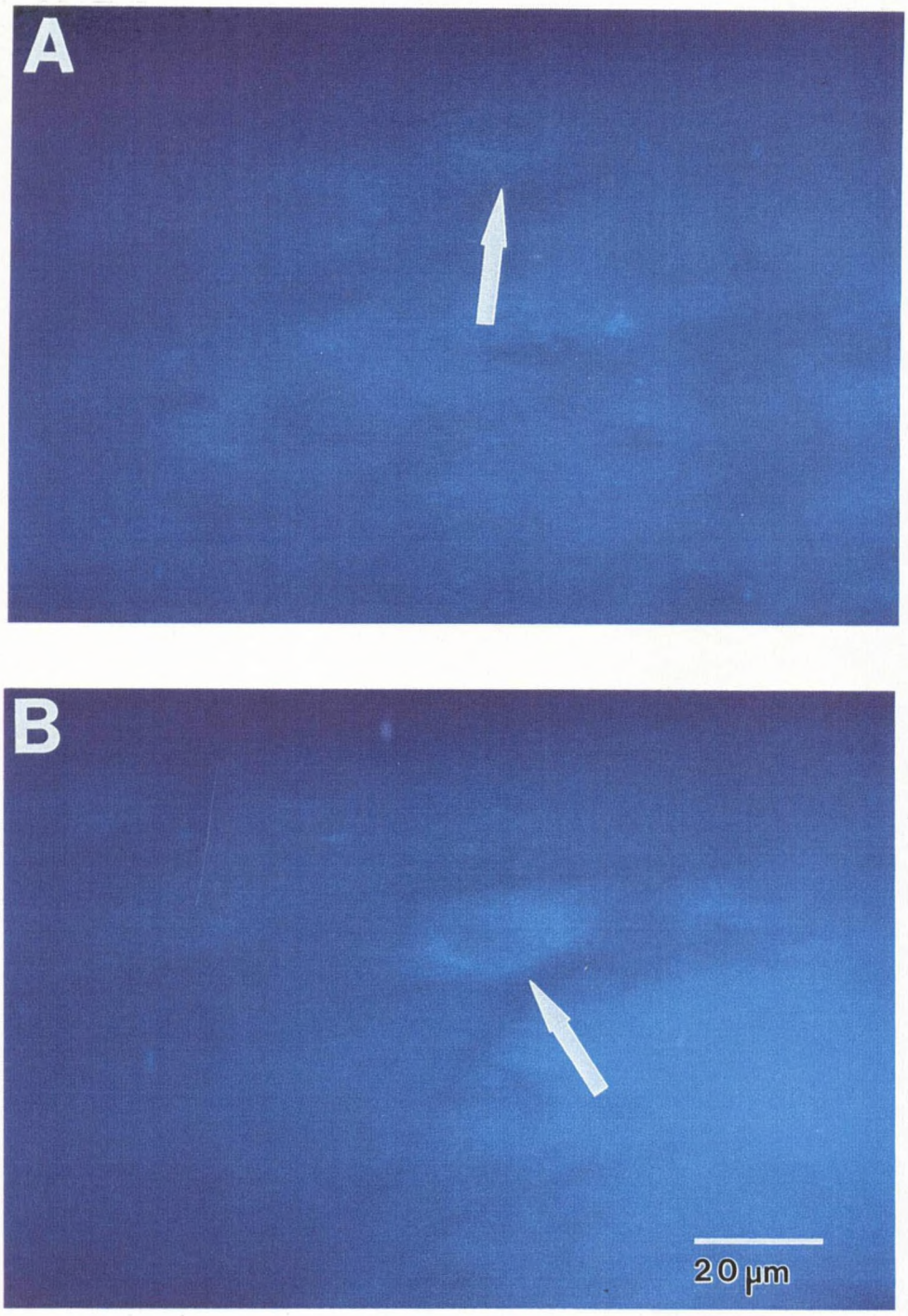

Figure 11. UV epifluorescent micrographs of representative SCG sections taken from FG-labeled rats. No specific labeling of neurons was apparent in any of the SCGs analyzed. However, rare, faint light-blue autofluorescing cells were seen (arrows). The absence of FG labeling in the SCG confirms results of the SCGX study showing GAP-43-ir processes found in the AP are not originating in the sympathetic ganglia. Magnification is 900X.

Vibratome sections of the trigeminal nerves and attached semilunar ganglia were also examined to further investigate the possibility that axons entering the AP are of sensory origin. No FG labeling of axons was observed in the trigeminal nerves, nor were labeled neural cell bodies seen in the semilunar ganglia of three of the four subjects (Figure 12). However, in the 14-day ADX animal some labeling was visible in both the trigeminal nerve and its ganglion. The bright gold labeling of the FG retrograde tracer appeared to be bilaterally localized to axons in the periphery of the trigeminal nerve, with sporadic staining of neural cell bodies being visible in the periphery of the semilunar ganglia. Because this staining was seen only in one animal, it was assumed to be artifactual. If this labeling was authentic the trigeminal nerves and ganglia of the 2-day animals should have also had FG-positive neurons since the dye would have had ample time to undergo endocytosis and retrograde transport back to the neural cell bodies. This interpretation is also supported by the possibility that the dye diffused back out the electrode tract into the surrounding tissue, thereby labeling the trigeminal nerve and ganglia which run adjacent to the pituitary gland. AP sections of the 14-day ADX animal did appear to show greater diffusion of FG from the injection site than was observed in the 2-day animals.

To examine the possibility that the innervation of the AP is of central origin, serial coronal Vibratome sections of the brain extending from approximately the lateral septal nuclei (1.00 mm posterior to bregma) through the pontine nuclei (7.60 mm posterior to bregma) were examined. Some staining appeared on the ventral surface of the brain and may have been the result of minor dye diffusion through the ME, a circumventricular organ. This labeling appeared to be localized within the processes and

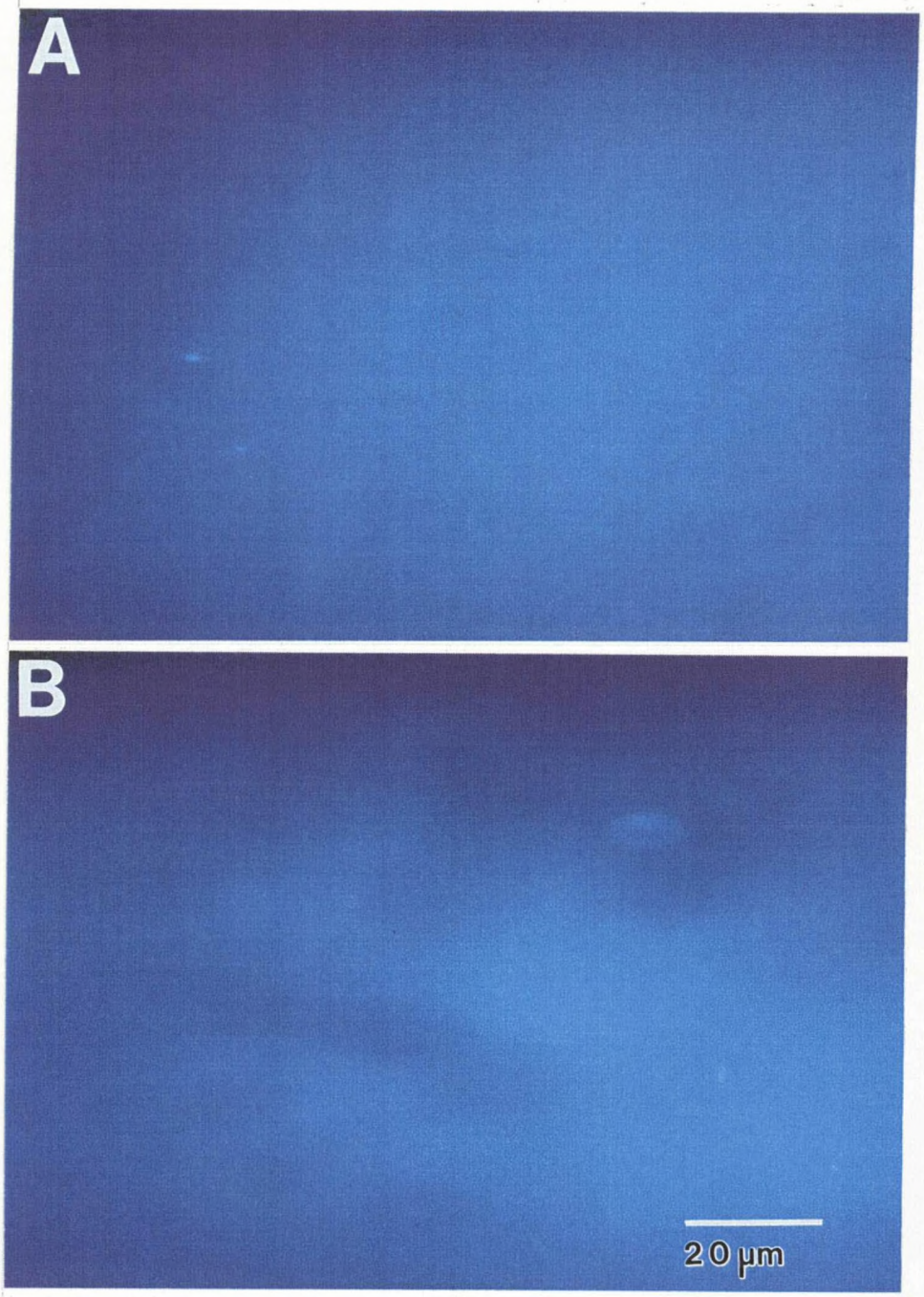

Figure 12. UV epifluorescent micrographs of representative semilunar ganglia **(A)** and trigeminal nerve **(B)** sections taken from FG-labeled rats. No specific labeling of neurons was apparent in most of the trigeminal ganglia and nerves analyzed. However, in the 14 day FG-ADX animal there appeared to be staining of axons in the periphery of the nerve and ganglia (not shown; see text). The absence of FG labeling in the trigeminals suggests that the GAP-43-ir processes are not sensory in origin. Magnification is 900X

cell bodies of fibroblasts. No labeling was apparent in the magnocellular neurosecretory neurons of the PVN or SON.

Analysis of the dorsal brain of all animals, except the 7-day ADX rat, revealed specific labeling of neural cell bodies within the medial habenular nuclei (MHb) (Figure 13). The dye was present bilaterally in both MHb nuclei with no labeling appearing outside of the habenular complex. Sporadic cell bodies were also labeled in the most medial portion of the lateral habenular nuclei adjacent to the MHb. However, this labeling was very sparse compared to the dense labeling present in the MHb. In the high magnification images it appeared that underlying cells were fluorescing through the superficial cell layer due to the broad focal plane of the microscope. Fewer cells appeared to be labeled in the peripheral MHb, with clearly visible neurites projecting away from the cell bodies (Figure 13).

No labeling was seen in the MHb of the 7-day ADX rat (Figure 14) in which the FG injection was shallow and confined to the fascia of the AP. This result supports the conclusion that labeling of the MHb in all other subjects was the result of retrograde transport of FG from the parenchyma of the AP.

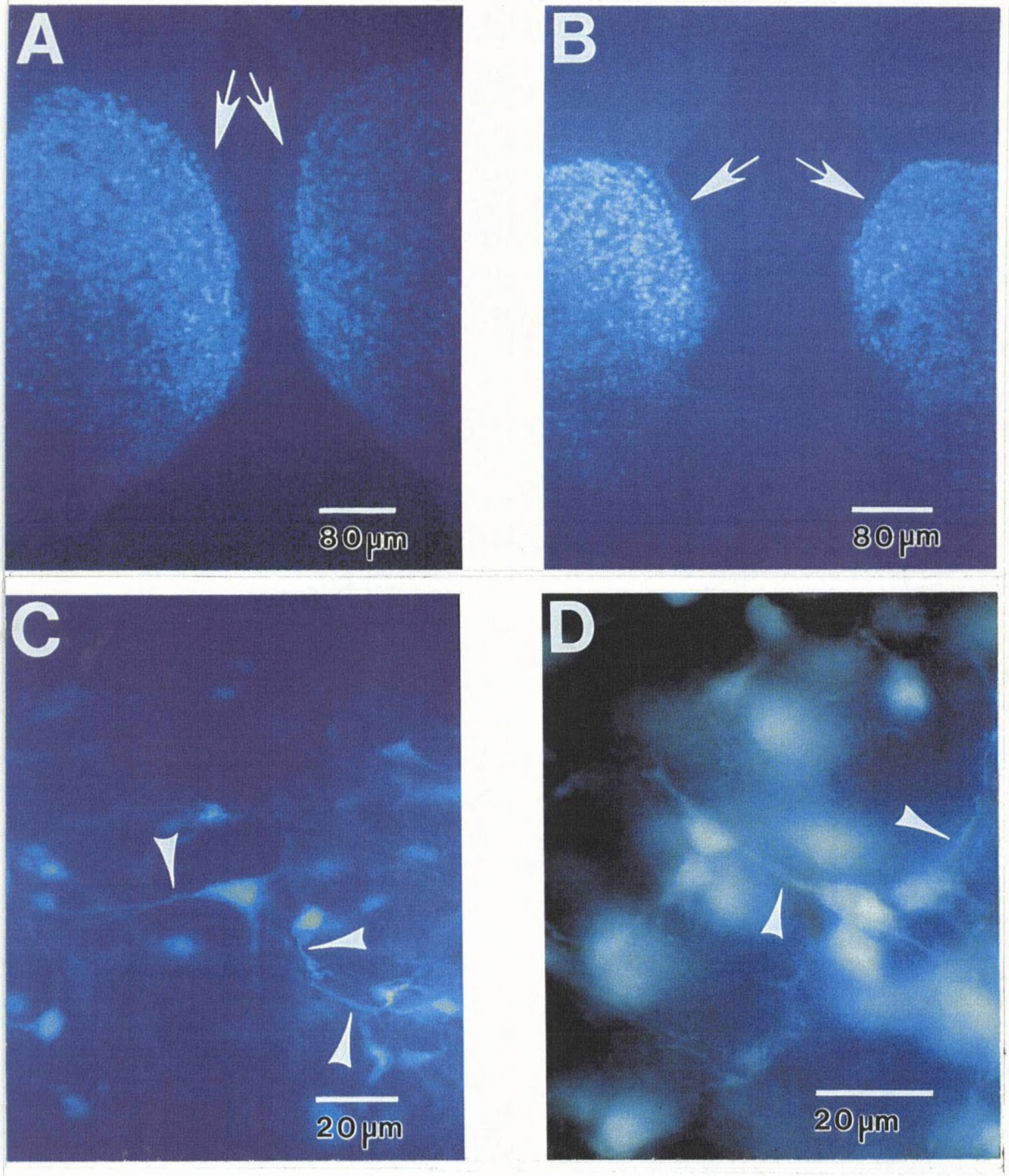

Figure 13. Representative UV epifluorescent micrographs of the MHb after FG iontophoresis into the rat AP. Low magnification micrographs **(A** and **B)** show the specific bilateral labeling of the MHb nuclei adjacent to the third ventricle. High magnification micrographs **(C** and **D)** were taken in the lateral region of the MHb where labeled neurites could be seen projecting from neural cell bodies (arrowheads). This labeling did not appear to be due to FG diffusion into the ventricular system since no labeling was present in the ependymal cell layer lining the third ventricle (arrows). Magnification is **(A)** 150X , **(B)** 150X , **(C)** 600X, **(D)** 900X.

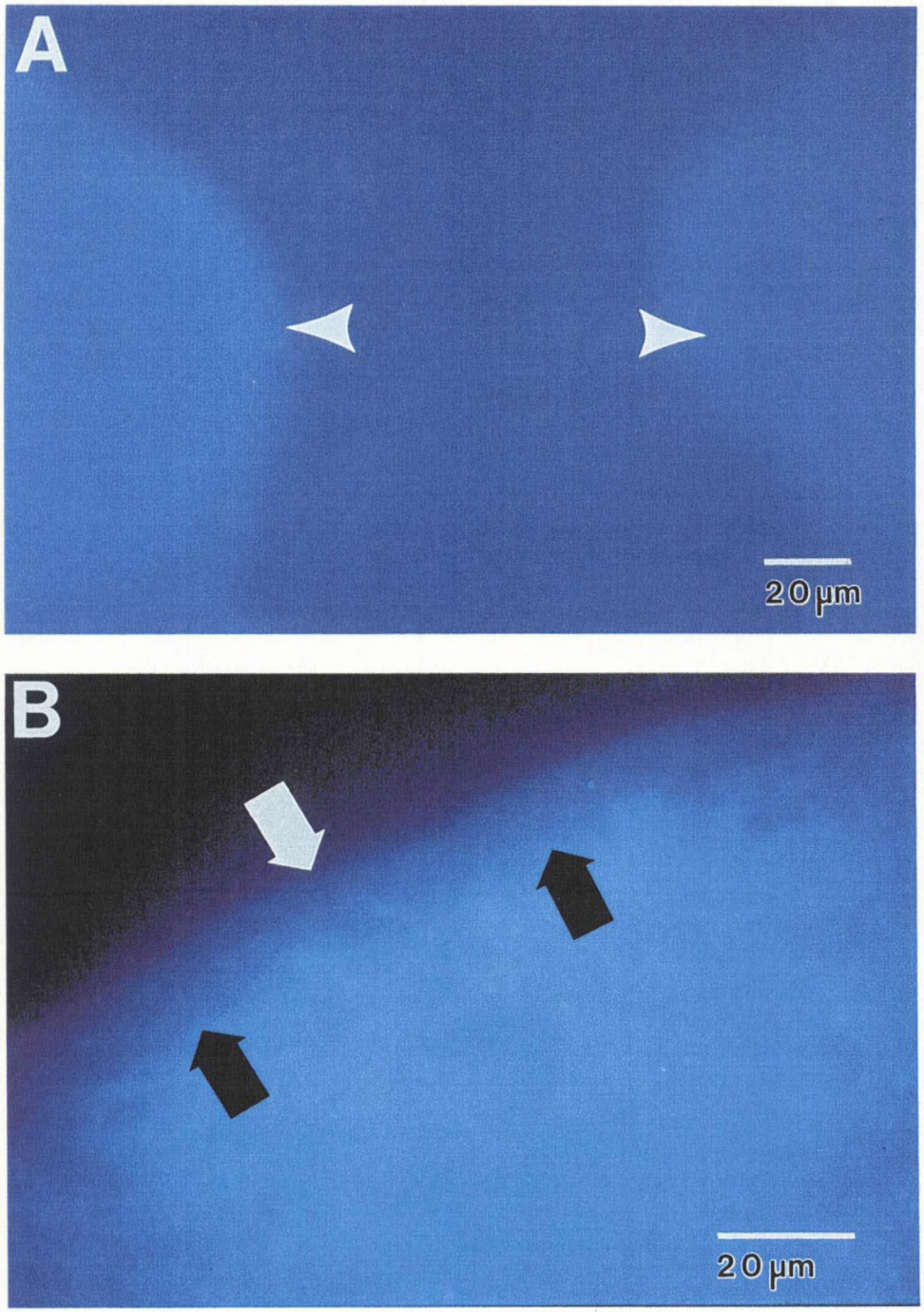

Figure 14. UV epifluorescent micrographs of the MHb of the 7 day FG-ADX rat. No labeling was seen in the MHb (**A**, arrowheads) or ependymal cell layer lining the third ventricle (**B**, arrows). The absence of retrograde labeling may have been due to the shallow injection of the FG into the superficial hypophysial fascia covering the AP. Magnification is **(A)** 600X , **(B)** 900X .

DISCUSSION

DiI Anterograde Tracing from the Trigeminal Nerves

Both forms of DiI appeared to have appropriately labeled trigeminal fibers as indicated in longitudinal sections of the nerve where extensive tracts of DiI-labeled axons were seen. However, very little staining was seen in coronal sections of the pituitary. In two of the ten animals used in this study a few fluorescent fibers were seen running along the periphery of the anterior lobe in the hypophysial fascia. These sporadic fibers are of potential interest since GAP-43-ir processes have been seen to occasionally enter the AP from its ventral and caudal surfaces, usually in association with the peripheral vasculature (Paden et al., 1994). However, the density of DiI labeled fibers was much less than the density of fibers seen when immunocytochemistry for GAP-43 was employed, and the fibers did not appear to penetrate a significant distance into the body of the AP. None the less, the possibility that these DiI positive fibers correspond to a subset of the GAP-43-ir processes cannot be excluded. Consistent with this possibility is the fact that fibers present in both the AP and CN V have been found to be immunoreactive for SP, CGRP, PACAP, and GAP-43 (Ju et al., 1993; Mikkelsen et al., 1995). In addition, the density of axonal processes immunoreactive for the sensory-related peptides SP and CGRP in the rat AP, like that of GAP-43-ir fibers, was seen to increase after ADX (Ju et al., 1993; 1994).

SCGX

The results of this experiment demonstrate that the GAP-43-ir axons found in the AP are not sympathetic afferents from the SCG, since removal of the SCG did not result in significant loss of GAP-43-ir in the AP. If the SCG was the source of innervation, a reduction in staining should have been apparent by one week after sympathectomy due to the onset of Wallerian degeneration and the phagocytosis of distal axonal processes following somal loss as reported by Romeo et al (1990). The fact that GAP-43-ir density is not decreased 8 days following SCGX therefore indicates that these fibers are nonsympathetic in nature.

This conclusion was confirmed by the results of the SCGX+ADX experiment, in which an increase in GAP-43-ir density was apparent 4 days after ADX in 8 day post-SCGX animals. SCGX did not appear to alter the ADX-induced upregulation of GAP-43 in the rat AP, with the majority of GAP-43-ir structures appearing as punctate clusters. Recent data (Paden et al., unpublished) suggesting that AP gland cells are capable of expressing GAP-43 makes it difficult to determine whether the increased punctate staining in SCGX+ADX rats is indicative of axonal collateral sprouting. However, long beaded GAP-43 positive fibers were also seen in the AP of SCGX+ADX rats (Figures 6, arrowheads) that could potentially be morphologically plastic. Collateral sprouting by these GAP-43-ir fibers is supported by Ju et al's (1993; 1994) work showing that SP/CGRP fibers of similar morphology sprout in the rat AP in response to ADX. Furthermore, the observation that GAP-43-ir density increases in the SCGX+ADX animal, in which levels of the sympathetic neurotransmitter norepinephrine are greatly

reduced in the hypothalamo-pituitary axis (Chiocchio et al., 1984), demonstrates that sympathetic outflow is not required for ADX-induced increases in GAP-43-ir to occur in the AP, whether due to axonal sprouting or expression of GAP-43 by gland cells.

Previous work in this laboratory has shown that the increase in GAP-43-ir in the AP of 4 day ADX animals is correlated with increased stimulation of the HPA axis and activation of corticotrophic secretory cells (Paden et al., 1998). The transient increase in GAP-43-ir 2 days after SCGX or sham-operation is probably similar in nature. This increase in GAP-43-ir density parallels the reported transient increase in circulating ACTH following SCGX or a sham operation (Romeo et al., 1990; Siaud et al., 1994). Plasma ACTH assays done by Romeo et al (1990) revealed a 12-fold increase in circulating ACTH in both SCGX and sham operated rats at 6 h after surgery. They further reported that ACTH levels declined dramatically in the sham-operated animals by 22 h after surgery, whereas in SCGX animals the ACTH level remained significantly elevated up to 54 h after ganglia removal. In both cases, ACTH concentration had returned to normal levels by 120 h after surgery.

Though SCGX appears to increase the release of prolactin while decreasing the release of follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, and growth hormone (Cardinali et al., 1987); SCGX has only a transient effect on plasma levels of ACTH (Romeo et al., 1991; Siaud et al., 1994). These reports are consistent with the present findings that the GAP-43-ir fibers seen in the AP do not arise from the SCG. This does not rule out the possibility that sympathetic input exists in the AP that is not involved in immediate control of circulating ACTH hormone levels.

In summary, our ability to visualize innervation of the AP using GAP-43 in SCGX, ADX, and SCGX+ADX rats has provided three novel findings with regard to innervation of the anterior lobe. First, they confirm that GAP-43-ir fibers in the AP do not arise from the sympathetic innervation of the HPA axis; second, they demonstrate that sympathetic outflow is not essential for increases in GAP-43-ir to occur; and third, they show that even transient activation of corticotrophs is correlated with increases in the density of GAP-43-ir.

DiI Retrograde Tracing from the AP

DiI retrograde tracing from the anterior lobe was done in an attempt to determine if the GAP-43-ir processes seen in the AP are of central origin. In all but a few of the intact, ADX, intact+NHX, and ADX+NHX brains, fibers were seen extending from the median eminence into the various brain regions listed in the results. In the labeled brains no association could be made between fiber density and whether or not the animal was intact or ADX. Very intense labeling of axons and neural cell bodies was seen throughout the hypothalamic region, especially in the areas making up the magnocellular neurosecretory system. Labeling was consistently seen in the PVN and SON of the hypothalamus which strongly suggests that the DiI applied to the AP was diffusing into the neural lobe and into the axolemma of vasopressinergic and oxytocinergic neurons.

It is known that axons of the magnocellular neurons in the SON do not project to the AP (Silverman and Zimmerman, 1983). Thus, the labeling of the SON appears to be an artifact due to dye diffusion into the NL. However, the PVN is known to contain five

distinct parvocellular subdivisions and all the projections of these neurons may not be known (Armstrong et al., 1980; Silverman and Zimmerman, 1983; Swanson and Sawchenko, 1983). Therefore, the labeling of any parvocellular PVN neurons may be real, while the labeling of any magnocellular PVN neurons is artifactual. Also in support of the possibility that at least part of the DiI labeling may be authentic is the fact that the periventricular nuclei (PE) were seen to contain axonal and somal staining. This is of interest since both CGRP and SP positive neuronal perikarya are present in the PE in the rat (Hokfelt et al., 1992; Kresse et al., 1992; Okamura et al., 1994).

In an attempt to reduce artifactual labeling arising from diffusion of DiI into the IL and NL, DiI was applied to the AP of NHX rats. SON staining persisted, making it impossible to use this model to confirm if PVN or PE neurons are the source(s) of the GAP-43-ir AP innervation. An additional indication that most if not all of the neuronal labeling by DiI was artifactual was the presence of diffuse staining in the median eminence, fornix, optic chiasm, infundibular stalk, and mammillothalamic tract. This labeling strongly suggests that DiI diffused from the AP to label these structures presumably by passing along blood vessels to the ME and hence to the ventricular system and brain.

Of particular interest was the faint labeling of axons seen extending from the hypothalamic region up to the edge of the medial habenular nuclei (MHb) in one of the ADX animals and two of the ADX+NHX animals. This staining was highly unexpected, but could be genuine. Cuello et al (1978) reported the existence of habenulo-interpeduncular-ventrotegmental SP projections extending from the MHb. It is possible that a subpopulation of these SP fibers could innervate the AP, a premise supported by

evidence that the MHb was seen to modulate the chronic stress response in rats (Murphy et al., 1996). This observation fits with previous studies from this laboratory showing that GAP-43-ir processes grow to contact corticotrophs in response to adrenalectomy (Paden et al., 1998), and also agrees with unpublished data showing that the density of GAP-43-ir is elevated in response to restraint stress.

In summary, this study proved inconclusive in defining the origin of the GAP-43-ir processes found in the AP. Therefore, the retrograde tracing study in living animals using Fluorogold was undertaken.

Fluorogold Retrograde Tracing from the AP

The relatively recent development of the fluorescent tracer dye, Fluorogold (FG), has provided neuroscientists with a powerful tool for retrograde labeling of neuronal pathways. Several unique characteristics of this dye make it better suited for labeling specific populations of neurons than previously available compounds. These properties include: (1) increased fluorescence, (2) extensive filling of dendritic processes, (3) high resistance to fading, (4) no uptake by intact fibers of passage, (5) no diffusion from the labeled neurons, (6) a long post-injection survival period, and (7) compatibility with other immunocytochemical or histochemical techniques (Schmued and Fallon, 1986). The aim of the present study was to determine whether or not axons present in the rat AP can be retrogradely traced back to their source(s) of origin.

The experimental findings demonstrated that the only structure consistently labeled was the medial habenular nuclei (MHb). Though one animal had peripheral

axons and cell bodies labeled in the trigeminal nerve and semilunar ganglia, this was not observed in any of the other rats. Furthermore, the labeling seen in the CN V and respective ganglia of the one animal could be accounted for by diffusion of FG out of the injection site into the adjacent nerve and ganglia.

The MHb and lateral habenular nuclei (LHb) were first described as distinct regions by Ramon y Cajal (1911). In 1979 the initial observation by Cajal was revisited by Herkenham and Nauta (1979) who divided the LHb into medial and lateral subdivisions. More recent analyses revealed that the habenula contains at least 15 distinct subnuclei (5 in the MHb, 10 in the LHb) (Andres et al., 1999); however, functional investigations of each subnuclei have yet to be completed.

The MHb lies on the medial diencephalic surface at the most posterior and dorsal part of the thalamus, forming small protrusions adjacent to the third ventricle (Scheibel, 1997). Together with the pineal gland this area is often considered a subdivision of the thalamus, known as the epithalamus. The functional importance of the habenula and its projections have remained enigmatic until recently. New evidence suggests that the MHb is involved in a wide variety of functions such as reproductive behaviors, central pain processing, nutrition, sleep-wake cycles, learning, and stress responses (Andres et al., 1999; Scheibel, 1997).

The major efferent tract from the habenula is the fasciculus retroflexus of Meynert, which projects principally to the interpeduncular nucleus (IPN) (Ramon y Cajal, 1911); but also documented are projections to the substantia nigra and ventral tegmental area, the medial raphe complex, and possibly the locus coeruleus and central gray (Scheibel, 1997). The major afferent innervation appears to come from neurons in

the preoptic area and septal region via the stria medullaris thalami. Thus, the habenular complex appears to serve as a critical modulatory relay station between the midbrain and the limbic forebrain.

Despite the recent increase in knowledge regarding the MHb, hardly any information exists on the precise circuits involved in mediating specific functions. However, it has been demonstrated that lesioning of the fasciculus retroflexus (FR) is capable of inducing morphological plasticity of peptidergic fibers within the interpeduncular nucleus of developing and adult rats (Artymyshyn and Murray, 1985; Eckenrode et al., 1987; Eckenrode et al., 1992). Furthermore, FR-lesioned rats also show elevated basal levels of stress hormones and evidence of stress-aggravated behavioral deficits in response to anxiogenic stimuli (Thornton and Bradbury, 1989), deficits in active avoidance learning (Thornton and Bradbury, 1989; Wilson et al., 1972), and exhibit less flexibility in choosing appropriate behavioral strategies when placed into a stressful environment (Thornton and Evans, 1982). Increased systemic levels of corticosterone after FR-lesions, indicative of increased corticotropin releasing factor (CRF) activity (Dunn and Berridge, 1990), is perhaps the cause of these behavioral effects. Taken together these data suggest that the MHb influences the activity of the HPA axis and may be involved in the modulation of ACTH release by the AP.

If the GAP-43-ir processes found in the AP do originate in the MHb, a novel, direct and highly plastic innervation of corticotrophs would be established that could be involved in the constellation of changes that occur in the HPA axis in response to stress-induced activation. However, until the precise relationship between plasticity of GAP-43-ir processes that contact corticotrophs (Paden et al., 1998) and activation of the HPA

axis is clearly defined, it is only possible to speculate regarding the potential functional significance of a projection from the MHb to the AP.

If the MHb does in fact innervate the AP the next goal would be to define the phenotype of the neurons in this pathway. Studies to date have shown that Substance P and cholinergic neurons present in the habenula (Cuello et al., 1978) project to the IPN via the FR; however, it remains uncertain if other sites of termination exist (Emson et al., 1976; Hong et al., 1976; Mroz et al., 1976). Though there does not appear to be any cholinergic innervation into the AP, studies done by Ju et al (1993; 1994) have shown a fairly dense innervation of SP fibers within the rat AP. Since no evidence was obtained in the present studies that SP positive sensory axons from the trigeminal ganglia innervate the AP, the possibility exists that SP positive axons found in the AP are projections from the MHb. If this is the case SP may act as an excitatory neurotransmitter (or modulator) in the AP as is the case in a number of well defined neural pathways (Leeman and Mroz, 1974).

The labeling of neuronal structures within the MHb does not appear to be an artifact. If the labeling was due to diffusion of the dye into the ventricular system the staining should appear in only the neurons most medial to the ventricle; however, this was not the case as labeling of neurons was seen throughout the entire MHb. Furthermore, the labeling was not present in the ependymal cell layer found lining the third ventricle (Figure 13), or in any other area juxtaposed to the ventricular system. It is possible that the FG labeling in the MHb could be an artifact due to the dye having entered the systemic circulation. However, the absence of neuronal labeling in both the subfornical area and the median eminence, two circumventricular organs, strongly

suggest that this did not occur. This is also supported by the fact that no labeling was seen in the PVN or SON of the magnocellular neurosecretory system whose axonal arbors are known to terminate outside the blood-brain-barrier, and therefore would have likely been labeled by any circulating FG. Finally, MHb neurons were completely filled with FG as expected following retrograde transport of the label.

To absolutely confirm the innervation of the AP by MHb neurons the next step that will be undertaken is anterograde tracing of MHb efferents. This experiment is currently in progress using the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L), a lectin extracted from the red kidney bean. If PHA-L labeled axons from the MHb are found in the AP, dual-label immunocytochemical staining for PHA-L and either GAP-43, SP, CGRP or PACAP will be carried out in an effort to discover the phenotype of these neurons.

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